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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
G01N 33/68, C12Q 1/48

A1

(11) International Publication Number: **WO 00/48002**

(43) International Publication Date: 17 August 2000 (17.08.00)

(21) International Application Number: PCT/GB00/00374

(22) International Filing Date: 9 February 2000 (09.02.00)

(30) Priority Data:
9902696.5 9 February 1999 (09.02.99) GB

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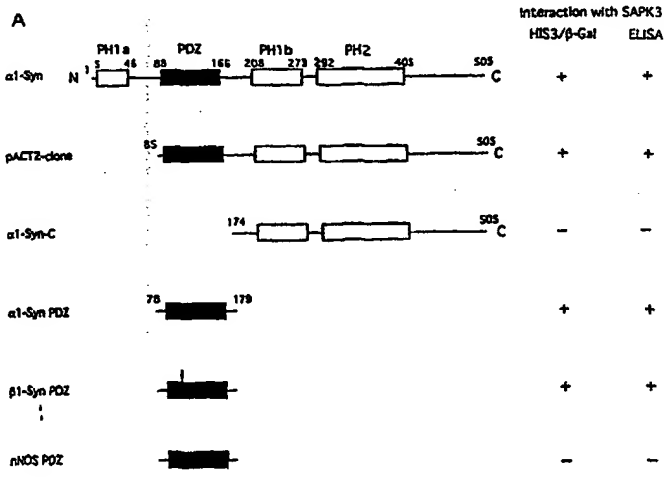
(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: SCREENING METHODS

(57) Abstract

A method of identifying a compound that is capable of modulating the interaction between (1) a protein kinase that is capable of (a) binding to a polypeptide comprising a PDZ domain and (b) phosphorylating the said polypeptide, and (2) the said polypeptide, wherein the method comprises the step of measuring the interaction between the said protein kinase and the said polypeptide. Also provided is a method of identifying a compound that is capable of modulating the phosphorylation of a polypeptide comprising a PDZ domain by a protein kinase wherein the protein kinase is capable of binding to and phosphorylating the said polypeptide, wherein the method comprises the step of measuring the phosphorylation of the said polypeptide by the said protein kinase. The protein kinase may be SAPK3. The polypeptide comprising a PDZ domain may be α1–syntrophin. The compounds identified may be useful in medicine.



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SCREENING METHODS

Stress-activated protein (SAP) kinases are mitogen-activated protein (MAP) kinase family members that are activated by cellular stresses, bacterial lipopolysaccharide and the cytokines intereukin-1 and tumour necrosis factor (reviewed in Cohen, P. (1997) *Trends Cell Biol.* **7,** 353-361). A major challenge in this field is to identify the physiological substrates and functions of each SAP kinase. SAPK1 [or c-Jun N-terminal kinase (JNK)] consists of a number of closely related isoforms that phosphorylate Ser63 and Ser73 in the activation domain of c-Jun, thereby increasing its transcriptional activity (Dérijard, B *et al* (1994) *Cell* **76,** 1025-1037; Gupta, S *et al* (1995) *EMBO J.* **15,** 2760-2770; Pulverer, B.J *et al* (1991) i **352,** 670-674). The same sites in c-Jun also become phosphorylated when cells are exposed to the stresses and cytokines that activate SAPK1 (Dérijard, B *et al* (1994); Pulverer, B.J *et al* (1991) *Nature* **352,** 670-6742, Pulverer, B.J *et al* (1991) *Nature* **352,** 670-674; Hibi, M *et al* (1993) *Genes Dev.* **7,** 2135-2148; Kyriakis, J.M *et al* (1994) *Nature* **369,** 156-160), suggesting that c-Jun is a physiological substrate for SAPK1. A second class of SAP kinase comprises SAPK2a (also called p38/RK/CSBPs) (Han, J *et al* (1994) *Science* **265,** 808-811; Rouse, J *et al* (1994) *Cell* **78,** 1027-1037; Lee, J.C *et al* (1994) *Nature* **372,** 739-746) and SAPK2b (Goedert, M *et al* (1997) *EMBO J.* **16,** 3563-3571) [also called p38β2 (Kumar, S *et al* (1997) *Biochem. Biophys. Res. Commun.* **235,** 533-538)] whose substrates include other protein kinases, such as MAP kinase-activated protein kinases-2 and -3 (MAPKAP-K2/K3) (Rouse, J *et al* (1994) *Cell* **78,** 1027-1037; Clifton, A.D *et al* (1996) *FEBS Lett.* **392,** 209-214), MAP kinase interacting protein kinases-1 and -2 (Mnk1/2) (Fukunaga, R. & Hunter, T. (1997) *EMBO J.* **16,** 1921-1933; Waskiewicz, A.J *et al* (1997) *EMBO J.* **16,** 1909-1920), p38-

regulated/activated protein kinase (PRAK) (New, L *et al* (1998) *EMBO J.* **17**, 3372-3384) and mitogen- and stress-activated protein kinases-1 and -2 (MSK1/2) (Deak, M *et al* (1998) *EMBO J.* 17, 4426-4441), as well as several transcription factors (Cohen, P. (1997) *Trends Cell Biol.* **7**, 353-361). Identification of physiological substrates of SAPK2a (p38) and SAPK2b (p38β2) is greatly facilitated because of the largely specific inhibition of these enzymes by the cell-permeant pyridinyl imidazole SB 203580 and related compounds (Lee, J.C *et al* (1994) *Nature* **372**, 739-746; Cuenda, A *et al* (1995) *FEBS Lett.* **364**, 229-233; Eyers, P.A *et al* (1998) Chem. Biol. **5**, 321-328; Gum, R.J *et al.* (1998) *J. Biol. Chem.* **273**, 15605-15610).

A third class of SAP kinase consists of the more recently identified SAPK3 (also called ERK6 and p38γ) (Mertens, S *et al* (1996) *FEBS Lett.* **383**, 273-276; Lechner, C *et al* (1996) *Proc. Natl. Acad. Sci. USA* **93**, 4355-4359; Li, Z *et al* (1996) *Biochem. Biophys. Res. Comm.* **228**, 334-340; Cuenda, A *et al* (1997) *EMBO J.* **16**,295-305) and SAPK4 (also called p38δ) (Goedert, M *et al* (1997) *EMBO J.* **16**, 3563-3571; Kumar, S *et al* (1997) *Biochem. Biophys. Res. Commun.* **235**, 533-538; Wang, X.S *et al* (1997) *J. Biol. Chem.* **272**, 23668-23674; Jiang, Y *et al* (1997) *J. Biol. Chem.* **272**, 30122-30128). The mRNAs encoding these enzymes are present in all mammalian tissues examined, with the mRNA encoding SAPK3 being most abundant in skeletal muscle (Mertens, S *et al* (1996) *FEBS Left.* **383**, 273-276; Lechner, C *et al* (1996) *Proc. Natl. Acad. Sci. USA* **93**, 4355-4359; Li, Z *et al* (1996) *Biochem. Biophys. Res. Comm.* **228**, 334-340). Expression of wild-type SAPK3 and an inactive mutant in the muscle cell line C2C12 enhanced and inhibited differentiation into myotubes, respectively (Lechner *et al*, 1996).

The amino acid sequence of SAPK3 is 60% identical to SAPK2a and SAPK2b and 47% identical to SAPK1. Like SAPK2, SAPK3 contains a TGY motif at positions 183 and 185 of full-length rat SAPK3 in the activation domain (which is TPY in SAPK1 and TEY in p42 and p44 MAP kinases) and subdomain VII is separated by six amino acids from the activation loop in subdomain VIII (as compared to eight residues in SAPK1 and >12 residues in any other MAP kinase family member).

SAPK3 and SAPK4 are not inhibited by SB 203580 (Goedert, M *et al* (1997) *EMBO J.* **16,** 3563-3571; Cuenda, A *et al* (1997) *EMBO J.* **16**,295-305) and consequently only little is known about their substrates. The substrate specificity of SAPK3 *in vitro* has been reported to be similar to that of SAPK2a, except that it was much less effective in activating MAPKAP-K2/K3 and (like SAPK1, but unlike SAPK2a) phosphorylated ATF2, a good substrate of SAPK3 *in vitro,* at Ser90, as well as at Thr69 and Thr71 (Cuenda *et al* (1997) *EMBO J.* **16**, 295-305). Stathmin has been proposed as a physiological substrate of SAPK4 (Parker, C.G *et al* (1998) *Biochem. Biophys. Res. Commun.* **249,** 791-796).

The syntrophins are a multigene family of intracellular dystrophin-associated proteins comprising three isoforms; α1, β1 and β2 as discussed, for example, in Peters *et al* (1997) "Differential association of syntrophin pairs with the dystrophin complex" *J Cell Biol* **138(1)**, 81-93, and Gee *et al* (1998) "Interaction of muscle and brain sodium channels with multiple members of the syntrophin family of dystrophin-associated proteins" *J Neurosci* **18(1)**, 128-137. Based on their domain organisation and association with neuronal nitric oxide synthase (nNOS), syntrophins are thought to function as modular adapters that recruit signalling proteins to the membrane *via* association with the dystrophin complex. Most

tissues express multiple syntrophin isoforms. In mouse gastrocnemius skeletal muscle, α1- and β1-syntrophin are concentrated at the neuromuscular junction but are also present on extrasynaptic regions of the sarcolemma. β1-Syntrophin is restricted to fast-twitch muscle fibres, which are the first type of fibre to degenerate in Duchenne muscular dystrophy. β2-Syntrophin appears to be restricted largely to the neuromuscular junction. The sarcolemmal distribution of α1- and β1-syntrophin suggests association with dystrophin and dystrobrevin, whereas all three syntrophins could potentially associate with utrophin at the neuromuscular junction. Dystrophin, dystrobrevin and utrophin are related proteins, all of which contain amino acid sequences homolgous to the dystrophin carboxy terminus, the region in dystrophin shown to bind syntrophins. Immunoisolation experiments indicated that utrophin complexes contain β1- and β2-syntrophins, whereas dystrobrevin complexes contain dystrophin and α1- and β1-syntrophins, although individual syntrophins do not appear to have intrinsic binding specificity for dystrophin, dystrobrevin or utrophin.

Each syntrophin isoform contains two pleckstrin homology (PH) domains, a syntrophin-unique (SU) domain and a PDZ domain, discussed further below. The PDZ domain is inserted in the first PH domain (Froehner, S.C *et al* (1987) *Cell Biol.* **104,** 1633-1646; Adams, M.E *et al* (1993) *Neuron* 11, 531-540; Lue, R.A *et al* (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9818-9822 (1994); Adams, M.E *et al* (1995) *J. Biol. Chem.* **270,** 25859-25865). The PDZ domains of syntrophins have been shown to bind to the PDZ domain of nNOS (Brenman, J.E *et al* (1996) *Cell* **84,** 757-767). The α1-subunits skM1 and SkM2 of voltage-gated sodium channels from skeletal muscle and heart (Trimmer, J.S *et al* (1989) *Neuron* 3, 33-49; Rogart, R.B *et al* (1989) *Proc. Natl. Acad. Sci. USA* **86,** 8170-

8174) have recently been shown to bind to the PDZ domain of α1-syntrophin through their carboxy-terminal sequences -KESLV [SkM1] or -RESIV [SkM2] (Rogart, R.B *et al* (1989) *Proc. Natl. Acad. Sci. USA* **86,** 8170-8174; Gee *et al* (1998) *J Neurosci* **18(1),** 128-137) corresponding to the proposed consensus binding sequence of (R/K/Q)E(S/T)XV-COOH (Gee *et al* (1998) *J Neurosci* **18(1),** 128-137; Schultz *et al* (1998) *Nature Struct Biol* **5(1),** 19-24). In skeletal muscle the interaction between SkM1 and α1-syntrophin has been proposed as a mechanism for anchoring voltage-gated sodium channels in the depths of the junctional folds of the post-synaptic membrane (Rogart, R.B *et al* (1989) *Proc. Natl. Acad. Sci. USA* **86,** 8170-8174; Schultz, J *et al* (1998) *Nature Struct. Biol.* **5,** 19-24).

The name PDZ domain is derived from the names of the first three proteins found to contain repeats of this domain (PSD-95, Drosophila discs large protein, and the zona occludens protein 1). PDZ domains are also known as DHF or GLGF domains. PDZ domains are reviewed, for example, in Ponting *et al* (1997) "PDZ domains: targeting signalling molecules to sub-membranous sites" *Bioessays* **19(6),** 469-479, Fanning & Anderson (1996) "Protein-protein interactions: PDZ domain networks" *Curr Biol* **6(11),** 1385-1388 and Cowburn (1997) "Peptide recognition by PTB and PDZ domains" *Curr Opin Struct Biol* **7(6),** 835-838. Examples of PDZ domains and PDZ-containing polypeptides are shown in Figures 7 and 8.

As discussed in Fanning & Anderson (1996), the PDZ domain sequence motif is about 80 to 90 amino acids long. PDZ domains can dimerise or bind to the carboxyl termini of unrelated proteins. These interactions contribute to the ability of PDZ domains to create networks associated

with the plasma membrane. Based on their binding specificities and sequence homologies, PDZ domains appear to fall into two classes, as discussed in Daniels *et al* (1998) "Crystal structure of the hCASK PDZ domain reveals the structural basis of class II PDZ domain target recognition" *Nature Struct Biol* **5(4)**, 317-325 and Songyang *et al* (1997) "Recognition of unique carboxyl-terminal motifs by distinct PDZ domains" *Science* **275(5296)**, 73-77. The C-terminal carboxylate binding loop of the PDZ domain is structurally conserved in both classes suggesting a generalised carboxylate binding motif (h-Gly-h) where h is a hydrophobic residue. The PDZ domains present in syntrophins are believed to fall into class I.

Doyle *et al* (1996) "Crystal structures of a complexed and peptide-free membrane protein-binding domain: molecular basis of peptide recognition by PDZ" *Cell* **85(7)**, 1067-1076 describes the crystal structures of the third PDZ domain from the synaptic protein PSD-95 in the presence and absence of its peptide ligand. The structures show that a four-residue C-terminal stretch (X-Thr/Ser-X-Val-COOH) engages the PDZ domain through antiparallel main chain interactions with a beta sheet of the domain. Recognition of the terminal carboxylate group of the peptide is conferred by a cradle of main chain amides provided by a Gly-Leu-Gly-Phe (GLGF) loop as well as by an arginine side chain. Specific side chain interactions and a prominent hydrophobic pocket may explain the selective recognition of the C-terminal consensus sequence.

The structures show that PDZ domains are compact globular $\alpha + \beta$ module of diameter 25-30 Å and contains six β strands (β1 to β6) and two α helices (α1 and α2). Main chain interactions between the bound peptide and strand β2 stabilise the bound peptide and sequence specificity is

conferred by domain interactions with the C-terminal carboxylate group of the bound peptide and the residues at positions 0 and -2 (relative to the C-terminus). Although several PDZ domains conserve all residues that contact the ligand in the crystal structure, no residues are absolutely conserved for all known PDZ domains, except the second glycine of the carboxylate binding loop. This may reflect the differing binding specificities of different PDZ domains.

Songyang *et al* (1997) "Recognition of unique carboxyl-terminal motifs by distinct PDZ domains" *Science* **275(5296)**, 73-77 discusses the optimal motifs recognised by nine different PDZ domains. One family of PDZ domains (Class I, according to the classification above), including those of the Discs Large protein, selected peptides with the consensus motif E-(S/T)-X-(V/I), at the carboxy terminus, where X represents any amino acid. Another family of PDZ domains (Class II, according to the classification above), including those of LIN-2, p55 and Tiam-1, selected peptides with hydrophobic or aromatic side chains at the carboxyl terminal three residues. These differences may be explained by reference to the crystal structures determined in Doyle *et al* (1996). Thus, class II PDZ domains differ from class I domains by formation of a second hydrophobic binding pocket and by preferentially binding polypeptides with hydrophobic or aromatic side chains at the carboxyl terminal three residues, whereas class I PDZ domains may preferentially bind polypeptides with the C-terminal consensus motif E-(S/T)-X-(V/I), where X represents any amino acid.

Staudinger *et al* (1997) *J Biol Chem* **272(51)**, 32019-32024 relates to specific interaction of the PDZ domain protein PICK1 with the COOH terminus of protein kinase C-alpha.

During vulval induction in *C. elegans*, the PDZ domain-containing protein LIN-7 is essential for localising the EGF receptor-like tyrosine kinase LET-23 to cell junctions by binding through its PDZ domain to the carboxy-terminal sequence -KETCL of LET-23 (Hoskins, R *et al* (1996) *Development* **122,** 97-111; Simske, J.S *et al* (1996) *Cell* **85**,195-204; Kaech, S.M *et al* (1998) *Cell* **94**,761-771). p70 S6 kinase has also been shown to bind through its carboxy-terminal sequence to the PDZ domain of neurabin, suggesting a mechanism for localising p70 S6 kinase to nerve terminals (Burnett, P.E *et al* (1998) *Proc. Natl. Acad. Sci. USA* 95, 8351-8356).

Here we show that a PDZ-domain-containing protein may be a substrate for a protein kinase and that phosphorylation of the PDZ-domain-containing protein may be dependent on the interaction of the carboxy-terminal sequence of the protein kinase with the PDZ domain of the PDZ-domain-containing protein. We show that such an interacting protein kinase and PDZ-domain-containing protein may colocalise *in vivo*. Compounds may thus be identified that are capable of modulating the phosphorylation state or interactions of a polypeptide comprising a PDZ domain. Such compounds may be useful in modulating physiologically relevant events and may therefore be useful in medicine or in research. Further, mutated polypeptides in which a said interaction or phosphorylation is altered may be useful in medicine or research.

A first aspect of the invention provides a method of identifying a compound that is capable of modulating the interaction between (1) a protein kinase that is capable of (a) binding to a polypeptide comprising a PDZ domain and (b) phosphorylating the said polypeptide, and (2) the said

polypeptide, wherein the method comprises the step of measuring the interaction between the said protein kinase and the said polypeptide.

A second aspect of the invention provides a method of identifying a compound that is capable of modulating the phosphorylation of a polypeptide comprising a PDZ domain wherein the protein kinase is capable of binding to and phosphorylating the said polypeptide, the method comprising the step of measuring the phosphorylation of the said polypeptide by the said protein kinase.

The said protein kinase may bind to the said polypeptide *via* the said PDZ domain. It will be appreciated that by binding *via* the said PDZ domain is included any binding for which the presence of a PDZ domain is necessary, even if other interactions between the said protein kinase and said polypeptide may also occur. It will be appreciated that binding *via* a PDZ domain may be characterised by the following feature: substantially no or reduced binding if the said PDZ domain is deleted, either partially or completely. Such binding of a protein kinase to a polypeptide comprising a PDZ domain, for example *via* the PDZ domain, may be detectable in a yeast two-hybrid screen, ELISA or co-immunoprecipitation experiment, as described in Example 1. It will be appreciated that binding *via* a PDZ domain may be further characterised by one or more of the following features: (1) substantially no or reduced binding if the carboxy-terminal four amino acids of the said protein kinase are deleted or mutated, as discussed further below, (2) substantially no or reduced binding in the presence of an antibody capable of binding to the carboxy-terminal 20, 16, 10, 8, 6 or 4 amino acids of the said protein kinase, (3) substantially no or reduced binding in the presence of a peptide

comprising an amino acid sequence corresponding to the carboxy-terminal 20, 16, 10, 8, 6 or 4 amino acids of the said protein kinase.

The interaction between the said protein kinase and the said polypeptide or the phosphorylation of the said polypeptide by the said protein kinase may be measured in the presence of more than one concentration of the compound (for example, in the presence of the compound and in the presence of substantially none of the compound). Thus, for example, the said protein kinase or said polypeptide, or both, may be exposed to more than one concentration of the compound in separate samples and the interaction or phosphorylation then measured in each said sample. The interaction or phosphorylation may be measured in the presence or absence of the compound, or in the presence of at least three concentrations of the compound, such that a dose response relationship may be derived, as well known to those skilled in the art. It will be appreciated that a concentration of compound at which the interaction or phosphorylation is decreased to 50% of that achieved in the absence of the compound, which may be termed the IC_{50} concentration, may be calculated from the dose response relationship.

The said protein kinase is preferably a cytoplasmic protein. Thus, the said protein kinase is preferably not a membrane-bound or embedded protein kinase, for example a receptor molecule that is embedded in a membrane *in vivo*. The said protein kinase is preferably a protein kinase with a C-terminal amino acid sequence that corresponds with the consensus sequence (T/S)-X-(V/I/L) or (E/D)-(T/S)-X-(V/I/L) or (R/Q/K)-(E/D)-(T/S)-X-(V/I/L), for example a protein kinase with the C-terminal amino acid sequence KETAL, KETPL, KETAV, KETPV, KESSL or KESSI. The one-letter amino acid code of the IUPAC-IUB Biochemical

Nomenclature Commission is used herein. In particular, X represents any amino acid.

The said protein kinase is preferably SAPK3 or a mammalian type-II activin receptor. Mammalian type-II activin receptors are transmembrane serine/threonine protein kinases of the TGFβ receptor superfamily with the carboxy-terminal sequences KESSL or KESSI (Matthews, L.S., & Vale, W.V. (1991) *Cell* **65**, 973-982; Attisano, L *et al* (1992) *Cell* **68**, 97-108). Activin type-I and type-II receptors may mediate activins' roles in regulating endocrine cells from the reproductive system, promoters of erythroid differentiation and in inducing axial mesoderm and anterior structures in vertebrates. Inhibins may have effects antagonistic to those of activins. BMP receptors may be involved in similar processes to TGFβ and activins, and particularly in bone growth and maintenance. TGFβs may be expressed in a wider range of tissues than other members of the superfamily, which may have more specialised roles.

A further aspect of the invention provides a method of identifying a compound that is capable of modulating the interaction between SAPK3 and a polypeptide comprising a PDZ domain wherein the method comprises the step of measuring the interaction between the said SAPK3 and the said polypeptide. As for the previous methods of the invention, the said interaction may be measured in the presence of more than one concentration of the compound, for example, in the presence of the compound and in the presence of substantially none of the compound, as described above. The said polypeptide comprising a PDZ domain is preferably capable of binding to SAPK3 *via* the said PDZ domain, which may be determined as discussed above.

A further aspect of the invention provides a method of identifying a compound that is capable of modulating the phosphorylation by SAPK3 of a polypeptide comprising a PDZ domain wherein the method comprises the step of measuring the phosphorylation of the said polypeptide by the said SAPK3. As for the previous methods of the invention, the said phosphorylation may be measured in the presence of more than one concentration of the compound, for example, in the presence of the compound and in the presence of substantially none of the compound, as described above. The said polypeptide comprising a PDZ domain is preferably capable of binding to SAPK3 *via* the said PDZ domain, which may be determined as discussed above.

The said polypeptide that comprises a PDZ domain may be a syntrophin, for example α1-syntrophin, β1-syntrophin or β2-syntrophin, preferably α1-syntrophin or may be a LIN-7 or a mammalian homologue of LIN-7 for example Veli1, 2 or 3 as described in Butz *et al* (1998) *Cell* **94**, 773-782 and as shown in Figure 10, or may be SAP90/PSD95 (EMBL codes X66474; rat or D50621; mouse, rat, human (see Ponting *et al* (1997)). The syntrophin may be phosphorylated on a serine or threonine residue, preferably on the residue equivalent to serine 193 and/or serine 201 of full-length human α1-syntrophin. It is preferred that the polypeptide that comprises a PDZ domain comprises a Class I PDZ domain. As discussed above, a PDZ domain may be classified as class I (as opposed to class II) on the basis of sequence comparisons with identified class I and class II domains and/or on the basis of the relative binding affinities with peptides corresponding to the respective consensus sequences given above (ie E-(S/T)-X-(V/I), at the carboxy terminus for class I and hydrophobic or aromatic side chains at the carboxyl terminal three residues for class II), as known to those skilled in the art. It is preferred that the polypeptide

that comprises a PDZ domain is not neuronal nitric oxide synthase (nNOS) or does not comprise a PDZ domain derivable from nNOS.

As discussed above, the amino acid sequence of SAPK3 is reported in Mertens *et al*, 1996 and is shown in Figure 6. SAPK3 is also known as ERK6 (Lechner *et al*, 1996) and p38γ (Li *et al*, 1996).

It will be appreciated that the term SAPK3 as used herein includes a polypeptide comprising the amino acid sequence shown in Figure 6 or identified as SAPK3 (also called ERK6 and p38γ) in Mertens, S *et al* (1996) *FEBS Lett.* **383**, 273-276, Lechner, C *et al* (1996) *Proc. Natl. Acad. Sci. USA* **93**, 4355-4359 or Li, Z *et al* (1996) *Biochem. Biophys. Res. Commun.* **228**, 334-340, or a variant, fragment, fusion or derivative thereof, or a fusion of a said variant or fragment or derivative. It is preferred that the said polypeptide is a protein kinase. It is preferred that the said polypeptide is a protein kinase that is capable of phosphorylating myelin basic protein and/or α1-syntrophin (preferably on the residue equivalent to amino acid Ser193 and/or Ser201 of full length α1-syntrophin). It is also preferred that the said polypeptide contains a TGY motif in the activation domain (which is TPY in SAPK1 and TEY in p42 and p44 MAP kinases) and/or that subdomain VII is separated by six amino acids from the activation loop in subdomain VIII (as compared to eight residues in SAPK1 and >12 residues in any other MAP kinase family member). It may also be preferred that, as for full-length wild-type SAPK3, the said polypeptide is not inhibited by SB 203580 (as discussed for SAPK3 and SAPK4 in Goedert, M *et al* (1997) *EMBO J.* **16**, 3563-3571; Cuenda, A *et al* (1997) *EMBO J.* **16**,295-305). It may further be preferred that the substrate specificity and/or other characteristics of the said polypeptide *in vitro* may be substantially as

reported in Cuenda *et al* (1997) *EMBO J.* **16**, 295-305; thus, the said polypeptide may phosphorylate ATF2, for example at Ser90, Thr69 and/or Thr71. It will be appreciated that the said polypeptide is not SAPK1, SAPK2a, SAPK2b or SAPK4.

It is particularly preferred, although not essential, that the variant or fragment or derivative or fusion of the said protein kinase, or the fusion of the variant or fragment or derivative has at least 30% of the enzyme activity of SAPK3 with respect to the phosphorylation of myelin basic protein. It is more preferred if the variant or fragment or derivative or fusion of the said protein kinase, or the fusion of the variant or fragment or derivative has at least 50%, preferably at least 70% and more preferably at least 90% of the enzyme activity of SAPK3 with respect to the phosphorylation of myelin basic protein. However, it will be appreciated that variants or fusions or derivatives or fragments which are devoid of enzymatic activity may nevertheless be useful, for example by interacting with another polypeptide. Thus, variants or fusions or derivatives or fragments which are devoid of enzymatic activity may be useful in a binding assay, which may be used, for example, in a method of the invention in which an interaction of SAPK3 (as defined above) with a polypeptide comprising a PDZ domain is measured.

By "variants" of a polypeptide we include insertions, deletions and substitutions, either conservative or non-conservative. In particular we include variants of the polypeptide where such changes do not substantially alter the activity of the said polypeptide, for example protein kinase activity if the said polypeptide is a protein kinase, for example SAPK3.

By "conservative substitutions" is intended combinations such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

It is particularly preferred if the SAPK3 variant has an amino acid sequence which has at least 65% identity with the amino acid sequence of SAPK3 shown in Figure 6, more preferably at least 70%, 71%, 72%, 73% or 74%, still more preferably at least 75%, yet still more preferably at least 80%, in further preference at least 85%, in still further preference at least 90% and most preferably at least 95% or 97% identity with the amino acid sequence defined above.

It is still further preferred if the SAPK3 variant has an amino acid sequence which has at least 65% identity with the amino acid sequence of the catalytic domain SAPK3 shown in Figure 6, more preferably at least 70%, 71%, 72%, 73% or 74%, still more preferably at least 75%, yet still more preferably at least 80%, in further preference at least 83 or 85%, in still further preference at least 90% and most preferably at least 95% or 97% identity with the amino acid sequence defined above. It will be appreciated that the catalytic domain of a protein kinase-related polypeptide may be readily identified by a person skilled in the art, for example using sequence comparisons as described below.

The percent sequence identity between two polypeptides may be determined using suitable computer programs, for example the GAP program of the University of Wisconsin Genetic Computing Group and it will be appreciated that percent identity is calculated in relation to polypeptides whose sequence has been aligned optimally.

The alignment may alternatively be carried out using the Clustal W program (Thompson *et al* (1994) *Nucl Acid Res* 22, 4673-4680). The parameters used may be as follows:

Fast pairwise alignment parameters: K-tuple(word) size; 1, window size; 5, gap penalty; 3, number of top diagonals; 5. Scoring method: x percent.

Multiple alignment parameters: gap open penalty; 10, gap extension penalty; 0.05.

Scoring matrix: BLOSUM.

It is preferred that the SAPK3 is a polypeptide which consists of the amino acid sequence of the protein kinase SAPK3 as shown in Figure 6 or naturally occurring allelic variants thereof.

It is preferred that the SAPK3 is a polypeptide that is capable of binding to a polypeptide comprising a PDZ domain, for example *via* the said PDZ domain. Whilst not bound by theory, it is believed that wild-type SAPK3 may bind to a PDZ domain, for example the PDZ domain of a syntrophin, for example α1-syntrophin by means of an interaction between the said PDZ domain and the C-terminal residues of the said SAPK3. Thus, it is preferred that the SAPK3 comprises a C-terminal amino acid sequence that corresponds with the consensus sequence (S/T)X(V/L) or (R/K/Q)E(S/T)X(V/L), for example KETXL, for example KETAL, KETPL, KETAV or KETPV.

The capability of the said SAPK3 with regard to binding a polypeptide comprising a PDZ domain may be measured by any method of detecting/measuring a protein/protein interaction, as discussed further below. Suitable methods include methods analagous to those discussed above and described in Example 1, for example yeast two-hybrid

interactions, ELISA or co-immunoprecipitation methods. Thus, the said SAPK3 may be considered capable of binding a polypeptide comprising a PDZ domain if an interaction may be detected between the said SAPK3 and the said polypeptide by a yeast two-hybrid interaction, ELISA or co-immunoprecipitation method, for example as described in Example 1.

The SAPK3 may, for example, be one of the following:

(1) a polypeptide with an amino acid sequence comprising or consisting of the amino acid sequence of SAPK3(1-363), which lacks the residues equivalent to residues 364 to 367 of full-length human or rat SAPK3, ie the carboxy-terminal four amino acids of full-length human or rat SAPK3, for example human or rat wild-type SAPK3,

(2) a polypeptide with an amino acid comprising or consisting of the amino acid sequence of L367VSAPK3, as described in Example 1, in which the residue equivalent to residue 367 of full-length human or rat SAPK3, ie the C-terminal leucine residue of human or rat wild-type SAPK3, is replaced by a valine residue,

(3) a polypeptide with an amino acid sequence comprising or consisting of the amino acid sequence of SAPK3, for example human or rat wild-type SAPK3, wherein at least the residues equivalent to the carboxy-terminal four amino acids of wild-type SAPK3 are missing or mutated, preferably such that the said polypeptide is not capable of binding to a PDZ domain, for example the PDZ domain of α1-syntrophin,

(3) a fusion polypeptide of glutathione-S-transferase (GST) or thioredoxin and any of the variants of SAPK3 described above, for example a fusion protein comprising the GST encoded by the GST sequence of the plasmid pEBG2T, or the thioredoxin encoded by the thioredoxin sequence of the plasmid pET32a (Novagen) as known to those skilled in the art. Examples

include GST-L367VSAPK3 and thioredoxin-SAPK3(1-363) as described in Example 1.

It will be appreciated that SAPK3 in which residues with the amino acid sequence of the most C-terminal four amino acids of wild-type SAPK3, for example human or rat SAPK3 are not present may not be capable of binding significantly to a PDZ-domain-comprising polypeptide, for example α1-syntrophin and may therefore be less preferred than some other SAPK3s for use in the screening methods of the invention. Thus, GST-SAPK3(1-363), as described in Example 1, may not be capable of significantly binding or phosphorylating α1-syntrophin and may therefore be less preferred than some other SAPK3s for use in the screening methods of the invention.

It will further be appreciated that a polypeptide (for example, SAPK3) in which residues with the amino acid sequence of the most C-terminal four amino acids of wild-type SAPK3, for example human or rat SAPK3, or with an other amino acid sequence that corresponds to the consensus sequence given above are not present at the C-terminus of the polypeptide but are present elsewhere in the polypeptide may be capable of binding a PDZ domain, for example the PDZ domain of α1-syntrophin. It is preferred that residues with the amino acid sequence of the most C-terminal four amino acids of wild-type SAPK3 or with an other amino acid sequence that corresponds to the consensus sequence given above are present at the C-terminus of the polypeptide.

It will be appreciated that the said SAPK3 may be capable of being activated, for example by being phosphorylated. It is preferred that such a SAPK3 is activated. SAPK3 may be activated by phosphorylation, in

particular by phosphorylation by SKK3 (Cuenda *et al* (1997) *EMBO J.* **16**, 295-305; also called MKK6 or MEK6), or a variant, fragment, fusion or derivative thereof, or a fusion of a said variant, fragment or derivative. The terms SKK3, MKK6 and MEK6 are well known to those skilled in the art (see, for example, Cuenda *et al* (1996) *EMBO J.* **15**, 4156-4164; Han *et al* (1996) *J. Biol. Chem.* **271**, 2886-2891; Moriguchi *et al* (1996) *J. Biol. Chem.* **271**,13675-13679; Raingeaud *et al* (1996) *Mol. Cell. Biol.* **16**, 1247-1255 and Stein *et al* (1996) *J. Biol. Chem.* **271**, 11427-11433) and includes, in particular, SKK3 as described and expressed in Cuenda *et al* (1996) *EMBO J.* **15**, 4156-4164 and in co-pending application WO98/15618. It is preferred that the said variant, fragment, fusion or derivative of SKK3, or a fusion of a said variant, fragment or derivative is a protein kinase, preferably a protein kinase capable of phosphorylating a polypeptide comprising an amino acid sequence corresponding to the consensus sequence Thr-Xaa-Tyr. Such variants of SKK3 may be functional equivalents of SKK3 and include SKK1.

Six chromatographically distinct SAP kinase kinases (SAPKKs or SKKs) have been identified in mammalian cells (Meier *et al* (1996) *Eur. J. Biochem.* **236**, 796-805; Cuenda *et al* (1996) *EMBO J.* **15**, 4156-4164). *In vitro*, SKK1 [also termed MKK4 (Dérijard *et al* (1995) *Science* **267**, 682-684), SEK1 (Sanchez *et al* (1994) *Nature* **372**, 794-798) and XMEK2 (Yashar *et al* (1993) *Mol. Cell. Biol.* **13**, 5738-5748)] activates all four groups of SAPKs (Sanchez *et al* (1994) *Nature* **372**, 794-798; Dérijard *et al* (1995) *Science* **267**, 682-684; Doza *et al* (1995) *FEBS Lett.* **364**, 223-228; Jiang *et al* (1996) *J. Biol. Chem.* **271**, 17920-17926; Cuenda *et al* (1997) *EMBO J.* **16**, 295-305), although SAPK2b and SAPK3 are phosphorylated less efficiently. SKK2 [also termed MKK3 (Dérijard *et al* (1995) *Science* **267**, 682-684)] and SKK3 (Cuenda *et al* (1996) *EMBO J.*

15, 4156-4164) [also called MKK6 (Han *et al* (1996) *J. Biol. Chem.* **271**, 2886-2891; Moriguchi *et al* (1996) *J. Biol. Chem.* **271**,13675-13679; Raingeaud *et al* (1996) *Mol. Cell. Biol.* **16**, 1247-1255) and MEK6 (Stein *et al* (1996) *J. Biol. Chem.* **271**, 11427-11433)] activate SAPK2a but not SAPK1, while SKK3 was the only detectable activator of SAPK3 induced by pro-inflammatory cytokines and stressful stimuli in human epithelial KB cells or human embryonic kidney 293 cells (Cuenda *et al* (1997) *EMBO J.* **16**, 295-305).

Activation of SAPK3 by SKK3 or SKK1 may be performed or assayed substantially as described in Cuenda *et al* (1997) *EMBO J.* **16**, 295-305, incorporated herein by reference or in WO98/15618.

It will be appreciated that SAPK3 may be activated by more than one SKK and that the said SKKs may act synergistically. Multiple stress/mitogen activated protein kinase kinases (SKK/MKKs) may activate a MAP/SAP kinase synergistically, which may be because they phosphorylate respectively the tyrosine residue and the threonine residue of the Thr-Xaa-Tyr motif, as described in co-pending application GB9824856.0.

"Variant", "fragment", "fusion" and "derivative" have equivalent meanings and preferences to those indicated earlier in relation to variants, fragments, fusions or derivatives of SAPK3.

It is preferred that an activating protein kinase, for example SKK3, is produced by a process involving recombinant DNA technology. Alternatively, an activating protein kinase may be derived from a cell in which the said activating protein kinase is endogenously expressed,

preferably a cell in which the said activating protein kinase is activated, as discussed above for SKKs/MKKs.

It will be appreciated that it may be necessary to activate a said activating protein kinase prior to use according to the invention. Bacterially expressed SKKs/MKKs may have some protein kinase activity, but typically this activity may be increased by about 1000-fold by treatment with an activating enzyme. Such activation may be carried out using the protein kinase MEKK.

Other protein kinases, for example mixed lineage kinase-2 (MLK-2), may activate other SKKs/MKKs (Cuenda & Dorrow (1998) *Biochem J* **333**, 11-15). At least five enzymes capable of activating SKK1, SKK2 and SKK3 *in vitro* and/or in cotransfection experiments have been identified, namely MEK kinase (MEKK) (Yan *et al* (1994) *Nature* **372**, 798-800; Lin *et al* (1995) *Science* **268**, 286-290; Matsuda *et al* (1995) *J. Biol. Chem.* **270**, 12781-12786; Blank *et al* (1996) *J. Biol. Chem.* **271**, 5361-5368), MAP kinase upstream kinase (MUK) (Hirai *et al* (1996) *Oncogene* **12**, 641-650), mixed lineage kinase-3 (MLK3) (Rana *et al* (1996) *J. Biol. Chem.* **271**, 19025-19028), TGFb-activated protein kinase-1 (TAK-1) (Moriguchi *et al* (1996) *J. Biol. Chem.* **271**,13675-13679) and the protooncogene Tpl2 (Salmeron *et al* (1996) *EMBO J.* **15**, 817-826).

Alternatively, the activating protein kinase may be a constitutively active protein kinase. Thus, for example, a mutant SKK/MKK in which the serine or threonine residue equivalent to serine 217 and/or serine 221 of MKK1 (preferably both) is/are each replaced by an aspartate or glutamate residue may be constitutively active (see, for example, Alessi *et al* (1994) *EMBO J* **13**, 1610-1619). Serines 217 and 221 or MKK1 are

phosphorylated in active MKK1, and mutation of these residues to alanine residues prevents activation and phosphorylation of MKK1. It is preferred that the activating protein kinase is a constitutively active protein kinase, for example constitutively active SKK3 in which both the said serine residues that may be phosphorylated in activated SKK3, for example that may be phosphorylated by MEKK, are replaced by aspartate residues.

It will be appreciated that the presence of a suitable source of a phosphate group, for example ATP, which may be present as the magnesium salt (MgATP), is required for the phosphorylation of a polypeptide by a protein kinase, as described, for example, in Example 1.

By "residue equivalent to residue [n] of full-length human SAPK3" is included the meaning that the amino acid residue that occupies a position in the native three dimensional structure of a protein kinase corresponding to the position occupied by the nth residue (counting from the amino-terminus) in the native three dimensional structure of human full-length SAPK3.

The residue equivalent to [n] of full-length human SAPK3 may be identified by alignment of the sequence of the polypeptide with that of full-length human SAPK3 in such a way as to maximise the match between the sequences. The alignment may be carried out by visual inspection and/or by the use of suitable computer programs, for example the GAP program of the University of Wisconsin Genetic Computing Group, which will also allow the percent identity of the polypeptides to be calculated. The Align program (Pearson (1994) in: Methods in Molecular Biology, Computer Analysis of Sequence Data, Part II (Griffin, AM and Griffin, HG eds) pp

365-389, Humana Press, Clifton). Thus, residues identified in this manner are also "equivalent residues".

It will be appreciated that in the case of truncated forms of SAPK3 or in forms where simple replacements of amino acids have occurred it is facile to identify the "equivalent residue".

The sequence for human SAPK3 is given, for example, in Goedert, M *et al* (1997) *EMBO J.* **16,** 3563-3571, Lechner, C *et al* (1996) *Proc. Natl. Acad. Sci. USA* **93,** 4355-4359 or Li, Z *et al* (1996) *Biochem. Biophys. Res. Commun.* **228,** 334-340.

By "PDZ domain" is meant a polypeptide or portion of a polypeptide that is capable of adopting a conformation characteristic of a PDZ domain, for example as determined in Daniels *et al* (1998) or Doyle *et al* (1996) above. The amino acid sequence of the said polypeptide or portion thereof may show significant sequence identity/conservative sequence substitution with an amino acid sequence identified as that of a PDZ domain. Thus, the amino acid sequence of the said polypeptide or portion thereof may have more than about 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% sequence identity with a PDZ domain, for example a PDZ domain of PSD-95, Drosophila discs large protein, or the zona occludens protein 1 or α1-syntrophin, preferably the third PDZ domain of PSD-95. The PDZ domain consensus sequence may comprise from about 60 to 120 70 to 110 amino acids, or 80 to 100 amino acids, but it will be appreciated that the amino acid sequence of the said polypeptide or portion thereof that has the said sequence identity with a PDZ domain may not be contiguous ie it may contain insertions or deletions. It is preferred that the said amino acid sequence is contiguous. The said polypeptide or portion thereof may

comprise the consensus amino acid sequence Gly-Leu-Gly-Phe or Gly-Leu-Gly-Ile or h-Gly-h, where h is a hydrophobic amino acid.

It is preferred that the said polypeptide that comprises a PDZ domain comprises a PDZ domain derivable from a syntrophin, LIN-7, Veli or SAP90/PSD95 protein, as discussed above or a variant, derivative, fragment or fusion thereof. It will be appreciated that the terms variant, derivative, fragment and fusion have meanings analogous to those described above in relation, for example, to SAPK3. It will further be appreciated that it is preferred that the said PDZ domain is capable of binding to a polypeptide comprising a C-terminal amino acid sequence corresponding to the consensus sequence (R/K/Q)-(D/E)-(T/S)-X-(V/I/L), in particular KETAL, KETPL, KETAV or KETPV, for example full-length SAPK3, for example from rat, human, mouse, rabbit or zebrafish, or full-length SAPK3 in which the C-terminal leucine is replaced by valine.

By a "syntrophin" is included a polypeptide comprising an amino acid sequence shown in Figure 9 or identified as a syntrophin in Ahn *et al* (1996) *J Biol Chem* **271**, 2724-2730, or a variant, fragment, fusion or derivative thereof, or a fusion of a said variant or fragment or derivative. By LIN-7 or Veli protein is included a polypeptide comprising an amino acid sequence shown in Figure 10 or identified as LIN-7 or a Veli protein in Butz *et al* (1998) *Cell* **94**, 773-782, or a variant, fragment, fusion or derivative thereof, or a fusion of a said variant or fragment or derivative. A syntrophin may have the domain structure with two pleckstrin homology (PH) domains, a syntrophin-unique (SU) domain and a PDZ domain, as discussed above. A syntrophin may be capable of interacting with dystrophin, dystrobrevin or utrophin, preferably *via* the carboxy-terminal

region of the dystrophin, dystrobrevin or utrophin, as described in Peters *et al* (1997) *J Cell Biol* **138(1)**, 81-93. A Veli protein may have a C-terminal PDZ domain and a more N-terminal domain that is homologous to the central domain of LIN-7 from *Caenorhabditis elegans*. A Veli protein may be capable of interacting with the polypeptide CASK (a protein related to MAGUKs; membrane-associated guanylate kinases) and/or the polypeptide Mint1 (a putative vesicular trafficking protein), as described in Butz *et al* (1998) *Cell* **94**, 773-782. The interactions of CASK, Mint1 and a Veli protein may not involve the PDZ domains of these polypeptides. A LIN-7 protein may be capable of interacting with the polypeptide LET-23 and/or the polypeptide LIN-2 from *C. elegans*, as described in Simske *et al* (1996) *Cell*, **85**, 195-204.

It is particularly preferred if the syntrophin, LIN-7, Veli or SAP90/PSD95 variant has an amino acid sequence which has at least 65% identity with the relevant amino acid sequence shown in Figure 9 or 10 or referred to in Ponting *et al* (1997), more preferably at least 70%, 71%, 72%, 73% or 74%, still more preferably at least 75%, yet still more preferably at least 80%, in further preference at least 85%, in still further preference at least 90% and most preferably at least 95% or 97% identity with the amino acid sequence defined above.

It is still further preferred if the syntrophin, LIN-7, Veli or SAP90/PSD95 variant has an amino acid sequence which has at least 65% identity with the amino acid sequence of the relevant PDZ domain shown in Figure 9, 10, 11 or 12 or referred to in Ponting *et al* (1997), more preferably at least 70%, 71%, 72%, 73% or 74%, still more preferably at least 75%, yet still more preferably at least 80%, in further preference at least 83 or

85%, in still further preference at least 90% and most preferably at least 95% or 97% identity with the amino acid sequence defined above.

The amino acid sequence of human PSD95 (sequence accession number U83192) is shown in Figure 11 and described in Stathakis *et al* (1997) *Genomics* **44(1)**, 71-82.

The comparison of amino acid sequences or three dimension structure (for example from crystallography or computer modelling based on a known structure) may be carried out using methods well known to the skilled man, as detailed below.

PDZ domains show conserved structural features and amino acid sequence similarities, as discussed above.

PDZ domains may be identified by alignment of the sequence of the polypeptide or portion thereof with that of known PDZ domains in such a way as to maximise the match between the sequences. The alignment may be carried out by visual inspection and/or by the use of suitable computer programs, for example the GAP program of the University of Wisconsin Genetic Computing Group, which will also allow the percent identity of the polypeptides to be calculated. The Align program (Pearson (1994) in: Methods in Molecular Biology, Computer Analysis of Sequence Data, Part II (Griffin, AM and Griffin, HG eds) pp 365-389, Humana Press, Clifton).

References for the sequence for PSD-95, Drosophila discs large protein, or the zona occludens protein 1 or α1-syntrophin are given, for example, in Ponting *et al* (1997) *BioEssays* **19(6)**, 469-479 and shown in Table 2.

Alignments of representative PDZ domain sequences are shown in Figures 7 and 8.

A further aspect of the invention provides a method of modulating the interaction between a protein kinase that is capable of binding to a polypeptide comprising a PDZ domain and is capable of phosphorylating the said polypeptide, and the said polypeptide, wherein a compound identified or identifiable by a screening method of the invention, as described above, is used.

A further aspect of the invention provides a method of modulating the phosphorylation of a polypeptide comprising a PDZ domain by a protein kinase wherein the protein kinase is capable of binding to and phosphorylating the said polypeptide, wherein a compound identified or identifiable by a screening method of the invention, as described above, is used.

As discussed above, the said protein kinase may bind to the said polypeptide *via* the said PDZ domain.

Preferences for the said protein kinase and the said polypeptide comprising a PDZ domain are as set out for earlier aspects of the invention, as appropriate. Thus, for example, the protein kinase may be a cytoplasmic protein kinase, for example SAPK3, and the polypeptide comprising a PDZ domain may be a syntrophin (for example as defined by the domain structure as shown in Figure 1), for example α1-syntrophin.

A further aspect of the invention is a method of measuring the protein kinase activity of SAPK3 wherein a polypeptide comprising a PDZ

domain that is capable of being phosphorylated by SAPK3 is used as a substrate. The said polypeptide may be, for example, a syntrophin, for example α1-syntrophin.

A further aspect of the invention is a method of identifying a compound capable of modulating the activity of SAPK3 wherein the phosphorylation of a polypeptide that is capable of being phosphorylated by SAPK3 and that comprises a PDZ domain is measured. As for previous methods of the invention, the said phosphorylation may be measured in the presence of more than one concentration of the compound, for example, in the presence of the compound and in the presence of substantially none of the compound, as described above. The said polypeptide comprising a PDZ domain is preferably capable of binding to SAPK3 *via* the said PDZ domain, which may be determined as discussed above.

Methods may be substantially as described in Examples 1 to 3 or, for example, as described below. Thus, preferred methods of the invention include the following methods:

A method of identifying a compound that inhibits the activity of SAPK3, the method comprising contacting a compound with SAPK3, as defined above (ie including a suitable variant, fragment, derivative or fusion thereof or a fusion of a variant, fragment or derivative thereof) and determining whether the activity of the said SAPK3 is reduced compared to the activity of the said SAPK3 in the absence of said compound, wherein the activity of the said SAPK3 is determined by measuring its ability to phosphorylate a polypeptide comprising a PDZ domain, for example the substrate may be α1-syntrophin.

A method of identifying agents able to influence the activity of SAPK3, said method comprising:

a) exposing a test substance to SAPK3 (as defined above) in the presence of a substrate for SAPK3;

b) detecting whether (and, optionally, to what extent) said substrate has been phosphorylated, wherein the said substrate comprises a PDZ domain, for example the substrate may be α1-syntrophin.

Use of SAPK3 (as defined above ie including a variant, fragment, derivative or fusion thereof or a fusion of a said variant, derivative or fragment thereof) in a screening assay for compounds which inhibit the activity of the said SAPK3 or which block the activation of said SAPK3 by SKK3 wherein the screening assay comprises the step of determining the ability of SAPK3 to bind to and/or phosphorylate a polypeptide that comprises a PDZ domain, for example α1-syntrophin. As for the previous methods of the invention, the said binding or phosphorylation may be measured in the presence of more than one concentration of the compound, for example, in the presence of the compound and in the presence of substantially none of the compound, as described above.

A further aspect of the invention is a method of phosphorylating a syntrophin, for example α1-syntrophin (as defined above) wherein SAPK3 is used. Thus, the method may comprise contacting a syntrophin with SAPK3 and a suitable phosphate donor. The syntrophin, for example α1-syntrophin, may be phosphorylated on the residues equivalent to serine 193 and/or serine 201 of full-length human α1-syntrophin. α1-Syntrophin (for example full-length human α1-syntrophin and ΔN-(78-505)-α1-syntrophin) may be phosphorylated on the residue equivalent to serine 193 and/or serine 201 of full-length human α1-syntrophin by a preparation

containing SAPK3 activity, as described in Example 1. The method may be performed *in vitro*, for example using purified or semi-purified components, or may be performed in a cell, for example a mammalian cell that is stimulated by a proinflammatory cytokine (for example Tumour Necrosis Factor (TNF)) and/or UV radiation and/or bacterial endotoxin/lipopolysaccharide (LPS) or another stimulator of SAPK3 activity. The method may comprise culturing a host cell comprising a recombinant polynucleotide or a replicable vector which encodes α1-syntrophin, and optionally also SAPK3, stimulating the cell, for example with a proinflammatory (for example Tumour Necrosis Factor (TNF)) and/or UV radiation and/or bacterial endotoxin/lipopolysaccharide (LPS) and isolating said polypeptide from said host cell. Methods of cultivating host cells and isolating recombinant proteins are well known in the art.

Such a method may be useful in investigating the effects of phosphorylation on the said syntrophin or in preparing a phosphorylated syntrophin. Such a phosphorylated syntrophin may be useful in research or in medicine. For example, a phosphorylated syntrophin may be useful in a method of identifying a phosphatase that is capable of dephosphorylating the said syntrophin.

A further aspect of the invention is a phosphorylated syntrophin, for example α1-syntrophin. The syntrophin, for example α1-syntrophin, may be phosphorylated on the residues equivalent to serine 193 and/or serine 201 of full-length human α1-syntrophin. A further aspect of the invention is a phosphorylated syntrophin obtainable by the above method of the invention. The said phosphorylated syntrophin may be substantially pure syntrophin or phosphorylated syntrophin when combined with other components *ex vivo*, said other components not being all of the

components found in the cell in which the said syntrophin is found.

A residue equivalent to residue [n] of full-length α1-syntrophin may be identified by methods analagous to those described above in relation to SAPK3. Sequences for full-length α1- syntrophin, β1-syntrophin and β2-syntrophin from mouse are given, for example, in Peters *et al* (1997) *J Cell Biol* **138(1)**, 81-93. Sequences for human α1- syntrophin, β1-syntrophin and β2-syntrophin may be given in, for example, Ahn *et al* (1996) *J Biol Chem* **271**, 2724-2730 and Ahn *et al* (1994) *PNAS* **91(10)**, 4446-4450 and are shown in Figure 12.

A further aspect of the invention provides a method of identifying a phosphatase that is capable of dephosphorylating a syntrophin wherein a phosphorylated syntrophin of the invention is used, the method comprising contacting the said phosphorylated syntrophin with a preparation that may comprise a said phosphatase and determining whether and to what extent the said phosphorylated syntrophin is dephosphorylated. The preparation may, for example, comprise a substantially polypeptide, which may be known to be capable of dephosphorylating other proteins or it may comprise a cell extract that may be unfractionated or fractionated using methods well known to those skilled in the art. Suitable methods for measuring dephosphorylation will be known to those skilled in the art.

A further aspect of the invention is a polypeptide comprising the amino acid sequence of mammalian, for example human or rat, SAPK3 or a fragment, variant, derivative or fusion thereof wherein the residue equivalent to glutamate 364 of full-length rat SAPK3 is replaced (by any residue, preferably aspartate) or missing (ie deleted) and/or the residue equivalent to threonine 365 of full-length rat SAPK3 is replaced (by any

residue, preferably by serine) or missing, and/or the residue equivalent to alanine 366 of full-length rat SAPK3 is replaced (by any residue) or is missing and/or the residue equivalent to leucine 367 of full-length rat SAPK3 is replaced (by any residue, preferably a hydrophobic residue, still more preferably valine) or is missing. The residue equivalent to each of the above residues may be identified by the method described above; it will be appreciated that such identification is typically trivial. It will be appreciated that a said polypeptide in which any of the above residues is missing ie deleted may be unable to bind a polypeptide comprising a PDZ domain, for example α1-syntrophin, *via* the PDZ domain. It will further be appreciated that a said polypeptide in which any of the residues equivalent to residues 364, 365 and 367 of full-length rat SAPK3 is replaced by a residue other than, respectively, aspartate, serine or a hydrophobic residue, preferably valine may be unable to bind a polypeptide comprising a PDZ domain, for example α1-syntrophin, *via* the PDZ domain. It will be appreciated that preferred polypeptides of the invention include polypeptides wherein the amino acid sequence of the said variant, fragment, fusion or derivative differs as described above from the amino acid sequence of a full length mammalian SAPK3 but does not otherwise differ from that of a full length mammalian SAPK3, for example human SAPK3. Further preferred polypeptides of the invention include a variant, fragment, fusion or derivative of SAPK3 wherein the said variant, fragment, fusion or derivative is not capable of binding to a polypeptide comprising a PDZ domain but is capable of phosphorylating MBP. It will be appreciated that the above polypeptides may be useful in investigating the interaction between the said polypeptide and a polypeptide comprising a PDZ domain. The above polypeptides may further be useful in investigating the mode of action of a compound identified by a screening method of the invention. Thus, a said

polypeptide may be useful in determining, for example, whether a compound that is capable of modulating the phosphorylation of a polypeptide comprising a PDZ domain by SAPK3, acts by modulating the interaction of SAPK3 with the said PDZ domain or by modulating any other interaction between SAPK3 and the said polypeptide comprising a PDZ domain.

A further aspect of the invention is a polypeptide comprising the amino acid sequence of mammalian, for example human or rat, syntrophin or a fragment, variant, derivative or fusion thereof wherein the residues equivalent to residues 1 to 77 of full-length human α1-syntrophin are deleted and/or the residues equivalent to residues 180 to 505 of full-length α1-syntrophin are deleted or the residues equivalent to residues 1 to 173 of full-length human α1-syntrophin are deleted or the residues equivalent to residues 1 to 102 of human full-length β1-syntrophin and/or the residues equivalent to residues 205 to the C-terminus of human full-length β1-syntrophin are deleted. Such polypeptides may be useful in, for example, investigating the physiological relevance of the interaction between SAPK3 and a syntrophin, for example α1-syntrophin.

A further aspect of the invention is a peptide comprising the amino acid sequence KETAL or KETPL wherein the said peptide is not full-length mammalian SAPK3 or a fusion thereof. The peptide may be KPPRQLGARVPKETAL, PKETAL, RVPKETAL, PKETPL or RVPKETPL. The peptide may be up to about 100, 80, 70, 60, 50, 40, 30, 20, 18, 16, 15, 14, 12, 10, 8 or 6 amino acids in length. The peptide may consist of or comprise contiguous residues derivable from SAPK3, for example rat or human SAPK3. The peptide may be capable of reducing, preferably substantially preventing, an interaction between full

length SAPK3, for example full length rat SAPK3 and α1-syntrophin, measured, for example, as described in Example 1. It will be appreciated that the peptide may comprise a covalent modification, for example it may be modified by biotinylation ie comprise a biotin group. Such a peptide may be useful in disrupting an interaction between, for example, SAPK3 and a polypeptide comprising a PDZ domain, for example α1-syntrophin, for example *in vivo*.

The above polypeptides or peptide may be made by methods well known in the art and as described below, for example using molecular biology methods or automated chemical peptide synthesis methods.

It will be appreciated that peptidomimetic compounds may also be useful. Thus, by "peptide" we include not only molecules in which amino acid residues are joined by peptide (-CO-NH-) linkages but also molecules in which the peptide bond is reversed. Such retro-inverso peptidomimetics may be made using methods known in the art, for example such as those described in Mézière *et al* (1997) *J. Immunol.* 159, 3230-3237, incorporated herein by reference. This approach involves making pseudopeptides containing changes involving the backbone, and not the orientation of side chains. Mézière *et al* (1997) show that, at least for MHC class II and T helper cell responses, these pseudopeptides are useful. Retro-inverse peptides, which contain NH-CO bonds instead of CO-NH peptide bonds, are much more resistant to proteolysis.

Similarly, the peptide bond may be dispensed with altogether provided that an appropriate linker moiety which retains the spacing between the Cα atoms of the amino acid residues is used; it is particularly preferred if the

linker moiety has substantially the same charge distribution and substantially the same planarity as a peptide bond.

It will be appreciated that the peptide may conveniently be blocked at its N- or C-terminus so as to help reduce susceptibility to exoproteolytic digestion.

A further aspect of the invention is a polynucleotide encoding a polypeptide of the invention. A still further aspect of the invention is a recombinant polynucleotide suitable for expressing a polypeptide of the invention. The polynucleotide may be a vector suitable for replication and/or expression of the polypeptide in a mammalian/eukaryotic cell.

The polynucleotide or recombinant polynucleotide may be DNA or RNA, preferably DNA. The polynucleotide may or may not contain introns in the coding sequence; preferably the polynucleotide is or comprises a cDNA.

Site-directed mutagenesis or other techniques can be employed to create single or multiple mutations, such as replacements, insertions, deletions, and transpositions, as described in Botstein and Shortle, "Strategies and Applications of *In Vitro* Mutagenesis," *Science*, **229:** 193-210 (1985), which is incorporated herein by reference. Polymerase chain reaction based methods of site-directed mutagenesis may be used, as well known to those skilled in the art, for example as described in Example 1.

By "suitable for expressing" is mean that the polynucleotide is a polynucleotide that may be translated to form the polypeptide, for example RNA, or that the polynucleotide (which is preferably DNA) encoding the

polypeptide of the invention is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. The polynucleotide may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by any desired host; such controls may be incorporated in the expression vector.

Characteristics of vectors suitable for replication in mammalian/eukaryotic cells are well known to those skilled in the art, and examples are given below. It will be appreciated that a vector may be suitable for replication in both prokaryotic and eukaryotic cells.

A variety of methods have been developed to operably link polynucleotides, especially DNA, to vectors for example *via* complementary cohesive termini. Suitable methods are described in Sambrook *et al* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

A desirable way to modify the DNA encoding a polypeptide of the invention is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491. This method may be used for introducing the DNA into a suitable vector, for example by engineering in suitable restriction sites, or it may be used to modify the DNA in other useful ways as is known in the art.

In this method the DNA to be enzymatically amplified is flanked by two specific primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

The DNA (or in the case of retroviral vectors, RNA) is then expressed in a suitable host to produce a polypeptide comprising the compound of the invention. Thus, the DNA encoding the polypeptide constituting the compound of the invention may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of the polypeptide of the invention. Such techniques include those disclosed in US Patent Nos. 4,440,859 issued 3 April 1984 to Rutter *et al*, 4,530,901 issued 23 July 1985 to Weissman, 4,582,800 issued 15 April 1986 to Crowl, 4,677,063 issued 30 June 1987 to Mark *et al*, 4,678,751 issued 7 July 1987 to Goeddel, 4,704,362 issued 3 November 1987 to Itakura *et al*, 4,710,463 issued 1 December 1987 to Murray, 4,757,006 issued 12 July 1988 to Toole, Jr. *et al*, 4,766,075 issued 23 August 1988 to Goeddel *et al* and 4,810,648 issued 7 March 1989 to Stalker, all of which are incorporated herein by reference.

The DNA (or in the case of retroviral vectors, RNA) encoding the polypeptide constituting the compound of the invention may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the

desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the polypeptide, which can then be recovered.

It will be appreciated that the host cell, for example a mammalian cell such as 293 cells as described in Example 1, may be stimulated, for example using a proinflammatory cytokine (for example Tumour Necrosis Factor (TNF)) and/or UV radiation and/or bacterial endotoxin/lipopolysaccharide (LPS), such that the SAPK3 polypeptide may be phosphorylated and/or activated in the host cell. The phosphoryated and/or activated polypeptide may then be recovered, if necessary in the presence of phosphatase inhibitors, for example microcystin, for example as described in Example 1. Recovery may entail purification on glutathione-Sepharose, as described in Example 1.

293 cells are human transformed primary embryonal kidney cells that may be obtained from the American Type Culture Collection (ATCC), 12301

Parklawn Drive, Rockville, Maryland 20852-1776; catalogue reference ATCC CRL 1573.

Many expression systems are known, including bacteria (for example *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

The vectors include a prokaryotic replicon, such as the ColE1 *ori*, for propagation in a prokaryote, even if the vector is to be used for expression in other, non-prokaryotic, cell types. The vectors can also include an appropriate promoter such as a prokaryotic promoter capable of directing the expression (transcription and translation) of the genes in a bacterial host cell, such as *E. coli*, transformed therewith.

A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention.

Typical prokaryotic vector plasmids are pUC18, pUC19, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA, USA) and p*Trc*99A and pKK223-3 available from Pharmacia, Piscataway, NJ, USA.

A typical mammalian cell vector plasmid is pSVL available from Pharmacia, Piscataway, NJ, USA. This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells.

An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3, TRP1, LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

The present invention also relates to a host cell transformed with a polynucleotide vector construct of the present invention. The host cell can be either prokaryotic or eukaryotic. Bacterial cells are preferred prokaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343). Preferred eukaryotic host cells include yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred mammalian host cells include human embryonic kidney 293 cells (see Example 1), Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, and monkey kidney-derived COS-1 cells available

from the ATCC as CRL 1650. Preferred insect cells are Sf9 cells which can be transfected with baculovirus expression vectors.

Transformation of appropriate cell hosts with a DNA construct of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of prokaryotic host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2110 and Sambrook *et al* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Transformation of yeast cells is described in Sherman *et al* (1986) *Methods In Yeast Genetics, A Laboratory Manual*, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* **275**, 104-109 is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA.

Electroporation is also useful for transforming and/or transfecting cells and is well known in the art for transforming yeast cell, bacterial cells, insect cells and vertebrate cells.

For example, many bacterial species may be transformed by the methods described in Luchansky *et al* (1988) *Mol. Microbiol.* 2, 637-646 incorporated herein by reference. The greatest number of transformants is consistently recovered following electroporation of the DNA-cell mixture suspended in 2.5X PEB using 6250V per cm at 25:FD.

Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* **194**, 182.

Successfully transformed cells, ie cells that contain a DNA construct of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an expression construct of the present invention can be grown to produce the polypeptide of the invention. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* 98, 503 or Berent *et al* (1985) *Biotech.* 3, 208. Alternatively, the presence of the protein in the supernatant can be detected using antibodies as described below.

In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an expression vector produce proteins displaying appropriate antigenicity. Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium.

A further aspect of the invention provides a method of making the polypeptide of the invention the method comprising culturing a host cell comprising a recombinant polynucleotide or a replicable vector which encodes said polypeptide, and isolating said polypeptide from said host

cell. Methods of cultivating host cells and isolating recombinant proteins are well known in the art.

A still further aspect of the invention provides a method of making and phosphorylating a syntrophin, for example α1-syntrophin, the method comprising culturing a host cell, preferably a eukaryotic cell, comprising a recombinant polynucleotide or a replicable vector which encodes said syntrophin and optionally SAPK3, stimulating the cell, for example with a proinflammatory cytokine (for example Tumour Necrosis Factor (TNF)) and/or UV radiation and/or bacterial endotoxin/lipopolysaccharide (LPS) and isolating said syntrophin from said host cell. Methods of cultivating host cells and isolating recombinant proteins are well known in the art. The host cell may be a mammalian cell that is stimulated by, for example, a proinflammatory cytokine (for example TNF) and/or UV radiation and/or bacterial endotoxin\lipopolysaccharide (LPS).

A further aspect of the invention is a polypeptide obtainable by the above methods of the invention.

A still further aspect of the invention provides an antibody reactive towards a polypeptide consisting of the amino acid sequence KPPRQLGARVPKETAL, PKETAL, RVPKETAL, PKETPL, RVPKETPL or ASGRRAPRTGLLELRAG wherein the said antibody is substantially non-reactive with other portions of SAPK3 or α1-syntrophin. Examples of such antibodies and of methods of preparing such antibodies are given in Example 1.

Antibodies reactive towards the said polypeptides may be made by methods well known in the art. In particular, the antibodies may be polyclonal or monoclonal.

Suitable monoclonal antibodies which are reactive towards the said polypeptide may be prepared by known techniques, for example those disclosed in "*Monoclonal Antibodies: A manual of techniques*", H Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and Applications", SGR Hurrell (CRC Press, 1982).

Techniques for preparing antibodies are well known to those skilled in the art, for example as described in Harlow, ED & Lane, D "Antibodies: a laboratory manual" (1988) New York Cold Spring Harbor Laboratory.

The following assays may be useful in a screening method as set out above. It will be appreciated that a said screening method may be useful in identifying a compound that may be used in medicine or in further characterising the interaction between a said protein kinase and a said polypeptide comprising a PDZ domain *in vitro* or *in vivo*.

Interaction of a compound with a protein kinase may be measured by measuring inhibition of the enzymatic activity of the protein kinase or by measuring the association/dissociation of the compound from the protein kinase. Methods of measuring the interaction of a compound with a protein are well known to those skilled in the art and include displacement assays, for example measuring displacement of a compound known to interact with the said protein kinase by the compound under test.

It will further be appreciated that the phosphorylation of a substrate polypeptide may be detected by means described herein other than by detecting a change in enzymatic activity of the substrate.

It will be appreciated that the phosphorylation of the chosen substrate may be measured using techniques known to those skilled in the art. For example, detection may be by using labelled (eg radiolabelled; ^{32}P or ^{33}P) phosphate in free solution or attached to the substrate, and comparing the amount associated with (or dissociated from) the substrate before and after the assay.

Some of the assay components may be localised on a surface, such as a blotting membrane, or an assay plate for ELISA etc, although the assay may be carried out in solution.

The use or methods may be performed *in vitro*, either in intact cells or tissues, with broken cell or tissue preparations or at least partially purified components. Alternatively, they may be performed *in vivo*. The cells tissues or organisms in/on which the use or methods are performed may be transgenic. In particular they may be transgenic for the protein kinase under consideration or for a further polypeptide, for example a polypeptide comprising a PDZ domain, for example α1-syntrophin.

It will be appreciated that screening assays which are capable of high throughput operation will be particularly preferred. This may require substantial automation of the assay and minimisation of the quantity of a particular reagent or reagents required for each individual assay. Examples may include cell based assays and protein-protein binding assays. An SPA-based (Scintillation Proximity Assay; Amersham

International) system may be beneficial, for example as described in Example 2. For example, beads comprising scintillant and a substrate polypeptide, for example α1-syntrophin or a peptide comprising the PDZ domain and phosphorylation site(s) of α1-syntrophin may be prepared. The beads may be mixed with a sample comprising ^{32}P- or ^{33}P-γ-labelled ATP, SAPK3 (as defined above) and with the test compound. Conveniently this is done in a 96-well format. The plate is then counted using a suitable scintillation counter, using known parameters for ^{32}P or ^{33}P SPA assays. Only ^{32}P or ^{33}P that is in proximity to the scintillant, ie only that bound to α1-syntrophin that is bound to the beads, is detected. Variants of such an assay, for example in which the substrate polypeptide is immobilised on the scintillant beads *via* binding to an antibody or antibody fragment, may also be used.

Other methods of detecting polypeptide/polypeptide interactions include ultrafiltration with ion spray mass spectroscopy/HPLC methods or other physical and analytical methods. Fluorescence Energy Resonance Transfer (FRET) methods, for example, well known to those skilled in the art, may be used, in which binding of two fluorescent labelled entities may be measured by measuring the interaction of the fluorescent labels when in close proximity to each other.

It will be appreciated that by "suitable" we mean that the said components in the method are those that have interactions or activities which are substantially the same as those of the said protein kinase, for example SAPK3 or the said polypeptide comprising a PDZ domain, for example α1-syntrophin, as the case may be but which may be more convenient to use in an assay. For example, fusions of SAPK3 or α1-syntrophin with another moiety, for example a GST portion, are particularly useful since

said fusion may contain a moiety, such as a GST portion, which may allow the fusion to be purified readily.

The enhancement or disruption of the interaction between a said protein kinase, for example SAPK3 and an interacting polypeptide comprising a PDZ domain as defined above, or suitable derivatives, fragments, fusions or variants can be measured *in vitro* using methods well known in the art of biochemistry and include any methods which can be used to assess protein-protein interactions. It will be appreciated that the methods described may be performed in cells. In a further embodiment the yeast two-hybrid system may be used.

It will be appreciated that the invention provides screening assays for drugs which may be useful in modulating, for example either enhancing or inhibiting, the activity of a protein kinase, for example SAPK3 or its interactions with or phosphorylation of a polypeptide comprising a PDZ domain, for example a syntrophin, such as α1-syntrophin. The compounds identified in the methods may themselves be useful as a drug or they may represent lead compounds for the design and synthesis of more efficacious compounds.

The compound may be a drug-like compound or lead compound for the development of a drug-like compound for each of the above methods of identifying a compound. It will be appreciated that the said methods may be useful as screening assays in the development of pharmaceutical compounds or drugs, as well known to those skilled in the art.

The term "drug-like compound" is well known to those skilled in the art, and may include the meaning of a compound that has characteristics that may make it suitable for use in medicine, for example as the active ingredient in a medicament. Thus, for example, a drug-like compound may be a molecule that may be synthesised by the techniques of organic chemistry, less preferably by techniques of molecular biology or biochemistry, and is preferably a small molecule, which may be of less than 5000 daltons. A drug-like compound may additionally exhibit features of selective interaction with a particular protein or proteins and be bioavailable and/or able to penetrate cellular membranes, but it will be appreciated that these features are not essential.

The term "lead compound" is similarly well known to those skilled in the art, and may include the meaning that the compound, whilst not itself suitable for use as a drug (for example because it is only weakly potent against its intended target, non-selective in its action, unstable, difficult to synthesise or has poor bioavailability) may provide a starting-point for the design of other compounds that may have more desirable characteristics.

A further aspect of the invention is a kit of parts useful in carrying out a method, for example a screening method, of the invention. Such a kit may comprise a protein kinase capable of interacting with a polypeptide comprising a PDZ domain, for example SAPK3 (as defined above) and a polypeptide comprising a PDZ domain (as defined above).

It will be understood that it will be desirable to identify compounds that may modulate the activity of the polypeptide *in vivo*. Thus it will be understood that reagents and conditions used in the method may be chosen such that the interactions between the said protein kinase, for example

SAPK3 and the polypeptide comprising a PDZ domain are substantially the same as between human SAPK3 and its interacting polypeptide comprising a PDZ domain, for example α1-syntrophin *in vivo*. It will be appreciated that the compound may bind to the protein kinase, for example SAPK3 or may bind to the polypeptide comprising a PDZ domain, for example α1-syntrophin.

A further aspect of the invention is a compound identifiable or identified by the said screening method. It will be appreciated that such a compound may be a modulation, for example an inhibitor of the protein kinase used in the screen and that the intention of the screen may be to identify compounds that act as modulators, for example inhibitors of the protein kinase, even if the screen makes use of a binding assay rather than an enzymic activity assay. It will be appreciated that the modulatory, for example inhibitory action of a compound found to bind the protein kinase may be confirmed by performing an assay of enzymic activity in the presence of the compound.

A still further aspect of the invention is a compound (or polypeptide or polynucleotide) of the invention for use in medicine.

The compounds may be administered in any suitable way, usually parenterally, for example intravenously, intraperitoneally or intravesically, in standard sterile, non-pyrogenic formulations of diluents and carriers. The compounds of the invention may also be administered topically, which may be of particular benefit for treatment of surface wounds. The compounds of the invention may also be administered in a localised manner, for example by injection.

A further aspect of the invention is a method of treating a patient with a muscle disease, for example muscular dystrophy or in need of modulation of phosphorylation of a protein comprising a PDZ domain or in need of modulation of signalling *via* an activin receptor or a voltage gated channel wherein a modulator of SAPK3 protein kinase activity or interaction with a polypeptide comprising a PDZ domain, for example a suitable compound of the invention, is used.

The modulator may be a polypeptide or a peptidomimetic compound (as described above) that is capable of disrupting the interaction between the said protein kinase (for example SAPK3) and a polypeptide comprising a PDZ domain (for example, α1-syntrophin). Such a polypeptide may have or comprise an amino acid sequence corresponding to the consensus sequence (R/K/Q)-(E/D)- (T/S)-X-(V/I/L) wherein X represents any amino acid, for example KETAL, KETPL, KETAV or KETPV. It is preferred that the polypeptide is less than about 100, 80, 70, 60, 50, 40, 30, 20, 18, 16, 14, 12, 10, 8 or 6 amino acids in length. The modulator may alternatively be an antibody (which term includes an antibody fragment, as well known to those skilled in the art) that is capable of binding to a polypeptide comprising an amino acid sequence corresponding to the consensus sequence (R/K/Q)-(E/D)- (T/S)-X-(V/I/L) wherein X represents any amino acid, for example KETAL, KETPL, KETAV or KETPV. Thus, it will be appreciated that an aspect of the invention is the use of a said polypeptide or antibody in medicine.

A further aspect of the invention is a method of disrupting the localisation of SAPK3 at the neuromuscular junction or at the sarcolemma of skeletal muscle wherein a compound identifiable by the method of the invention or a polypeptide or antibody as defined above is used.

A further aspect of the invention is the use of a said polypeptide or antibody in the manufacture of a medicament for use in treating a patient in need thereof, for example a patient with a muscular disease, for example muscular dystrophy, or in need of modulation of phosphorylation of a protein comprising a PDZ domain or in need of modulation of signalling *via* an activin receptor or a voltage gated channel or in need of disrupting the localisation of SAPK3 at the neuromuscular junction or at the sarcolemma of skeletal muscle. The patient may be human.

The present invention will now be described in more detail with reference to the following non-limiting Figures and Examples.

Figure Legends.

Figure 1: Interactions between SAPK3 and α1-syntrophin

(A) Interaction of SAPK3 with the PDZ domain of α1-syntrophin. Binding of GAL4 fusion constructs of human α1-syntrophin, the PDZ domain of human β1-syntrophin and the PDZ domain of human neuronal nitric oxide synthase (nNOS) to rat SAPK3 was tested in the yeast two-hybrid system. Interactions were measured by the activity of the reporter genes HIS3 and β-Gal. HIS3 activity was judged by growth in medium lacking histidine in the presence of 25 mM 3-AT and β-Gal activity was determined from the time taken for the colonies to turn blue in X-Gal filter lift assays performed at room temperature: (+), 90-240 min; (-), no significant β-Gal activity. *In vitro* binding of SAPK3 to the PDZ domain-containing proteins was tested by ELISA. The two SAPK3-interacting clones isolated in the yeast two-hybrid screen (shown as pACT2) encoded residues 85-505 of human β1-syntrophin.

(B) Interaction of human α1-syntrophin with full-length rat SAPK3(1-367), but not with SAPK3(1-363).

Figure 2: Phosphorylation of α1-syntrophin by SAPK3 is dependent on the carboxy-terminal four amino acids of SAPK3

(A) GST-α1-syntrophin phosphorylated by SAPK3 was digested with trypsin and the resulting phosphopeptides chromatographed on a C_{18} column (see Methods). The two major tryptic phosphopeptides were shown to correspond to residues 198-207 and 178-197 and to be phosphorylated at Ser193 and Ser201, respectively. The acetonitrile gradient is shown by the broken line. (B) GST-α1-syntrophin (1 μM) or MBP (1 μM) was phosphorylated for the times indicated with 0.2 U/ml of either GST-SAPK3(1-367) or GST-SAPK3(1-363). The results are shown as means ± S.E.M. from three experiments. (C) Full-length GST-SAPK3 (0.2 U/ml) was incubated for 30 min at room temperature with the indicated concentrations of an antibody raised in sheep against the synthetic peptide KPPRQLGARVPKETAL which corresponds to residues 352-367 of rat SAPK3 (open symbols) (20) or with sheep IgG (closed symbols). The SAPK3 was then assayed in duplicate for 10 min with GST-α1-syntrophin (circles) or MBP (triangles). Substrate phosphorylation is plotted as a percentage of that measured in the absence of antibody. (D) GST-α1-syntrophin (filled bars) or MBP (open bars), each at 1 μM, were incubated for 30 min at room temperature with synthetic peptides (300 μM) corresponding to the C-terminal 6 or 8 amino acids of rat SAPK3. GST-SAPK3 was added to 0.2 U/ml and after 10 min the assays were initiated with Mg[γ^{32}P]ATP. Substrate phosphorylation is plotted as a percentage of that measured in the absence of each peptide. The concentration of each peptide required to inhibit

GST-α1-syntrophin phosphorylation by 50% was 30 μM. The results are shown as the means ± S.E.M. from a single experiment. The assays in (C) and (D) were carried out in duplicate and such results were obtained in two further experiments in each case.

Figure 3: Binding of L367V SAPK3 to α1-syntrophin and phosphorylation of α1-syntrophin by L367VSAPK3

(A) *In vitro* binding of wild-type SAPK3 and L367VSAPK3 to α1-syntrophin as determined by ELISA. (B,C) GST-α1-syntrophin (1 μM) [B] or MBP (1 μM) [C] was phosphorylated for the times indicated with 0.2 U/ml of either wild-type GST-SAPK3 or GST-V367LSAPK3.

Figure 4: Localization of SAPK3, α-bungarotoxin and α1-syntrophin in rat skeletal muscle

Sections of semitendinous muscle were double- or triple-stained with tetramethylrhodamine α-bungarotoxin (in red) (A), anti-SAPK3 serum R5 visualized with fluorescein-avidin D (in green) (B) and anti-α1-syntrophin serum SYN17 visualized with AMCA-streptavidin (in blue) (C). α-Bungarotoxin, SAPK3 and α1-syntrophin are present at the neuromuscular junction (A-C). SAPK3 and α1-syntrophin are also present throughout the sarcolemma (B,C). Scale bar: 17μm.

Figure 5: Co-immunoprecipitation of SAPK3 with α1-syntrophin

Lysates from COS cells transfected with rat SAPK3 alone and double-transfected with SAPK3 and human α1-syntrophin were immunoprecipitated with anti-α1-syntrophin serum TROPHA. Total cell lysates and immunoprecipitates (marked IP) were immunoblotted with anti-α1-syntrophin and anti-SAPK3 antibodies. α1-Syntrophin-

immunoreactive bands were present in double-transfected (marked SAPK3 +α1-syntrophin) cell lysates and immunoprecipitates (arrows). SAPK3-immunoreactive bands were detected in single-(marked SAPK3) and double-transfected (marked SAPK3 +α1-syntrophin) cell lysates (arrow head). SAPK3 was detected as an immune complex with α1-syntrophin in double-transfected cell lysates (arrow head), but not in cells transfected with SAPK3 alone. The strong band in the lanes marked IP corresponds to the IgG heavy chain.

Figure 6: Amino acid and nucleotide coding sequence of rat SAPK3

Figure 7: Alignment of representative PDZ domains (from Ponting *et al* (1997) *Bioessays* **19(6)**, 469-479)

Figure 8: Alignment of class I and class II PDZ domains (from Daniels *et al* (1998) *Nature Struct Biol* **5(4)**, 317-325)

Figure 9: Cloning, sequence and domain strucutre of murine β1-syntrophin (from Peters *et al* (1997) *J Cell Biol* **138(1)**, 81-93)

Figure 10: Characterisation of Velis (vertebrate LIN-7 homologs) (from Butz *et al* (1998) *Cell* **94**, 773-782)

Figure 11: Amino acid sequence of human PSD95 (Accession number U83192)

Figure 12: Amino acid sequences of human α1-syntrophin, β1-syntrophin and β2-syntrophin (Accession numbers U40571, L31529 and U40572 respectively)

Example 1: Stress-activated protein kinase-3 interacts with the PDZ domain of α-syntrophin: a mechanism for specific substrate recognition.

Mechanisms for selective targeting to unique subcellular sites play an important role in determining the substrate specificities of protein kinases. Here we show that stress-activated protein kinase-3 [(SAPK3), also called ERK6 and p38γ], a member of the mitogen-activated protein kinase family that is abundantly expressed in skeletal muscle, binds through its carboxy-terminal sequence -KETXL to the PDZ domain of α1-syntrophin. SAPK3 phosphorylates α1-syntrophin *in vitro* and phosphorylation is dependent on binding to the PDZ domain of α1-syntrophin. In skeletal muscle SAPK3 and α1-syntrophin co-localise at the neuromuscular junction and both proteins can be co-immunoprecipitated from transfected COS cells. Phosphorylation of a PDZ domain-containing protein by an associated protein kinase is a novel mechanism for determining both the localisation and the substrate specificity of a protein kinase.

Experimental Procedures.

Materials. Full-length human α1-syntrophin was obtained by PCR from human skeletal muscle cDNA. It was subcloned into pACT2 (Stratagene) for yeast two-hybrid screening or pGEX4T-1 (Pharmacia) for bacterial expression as a GST-fusion protein. α1-Syntrophin(78-179) and α1-syntrophin(174-505) were produced by PCR, as were human β1-syntrophin(103-204) and human neuronal nitric oxide synthase(9-108). Expression and activation of rat GST-SAPK3 have been described (23). Rat SAPK3(1-363) was produced by PCR and subcloned into pGEX4T-1 for expression as GST-fusion protein. For some experiments SAPK3(1-

363) and SAPK3(1-367) were subcloned into the yeast two-hybrid vector pAS2-1 (Stratagene) or the thioredoxin-fusion protein vector pET32a (Novagen). Site-directed mutagenesis was used to produce L367VSAPK3, followed by subcloning into pGEX4T-1 and expression as a GST-fusion protein. All constructs were verified by DNA sequencing. Expression and activation of recombinant MAP kinase, SAPK2a, SAPK2b and SAPK4 have been described (10).

Yeast two-hybrid system screening. Yeast two-hybrid screening (27) was performed using an adult human brain expression library (Clontech) containing cDNAs fused to the GAL4 transactivation domain of pACT2 and rat SAPK3 DNA (20) subcloned into vector pAS2-1 which contains the GAL4 DNA binding domain. The plasmids were transformed into Y190 yeast cells and positive clones were selected on triple-minus plates (Leu-, Trp-, His-) + 25 mM 3-aminotriazole (3-AT) and assayed for β-galactosidase activity. Two million clones were screened and two positives obtained. Positive clones were co-transformed with either the bait vector or the original pAS2-1 (used as a control) into yeast to confirm the interaction. All the constructs that were used in other interaction experiments were from PCR products subcloned into pAS2-1 or pACT2 and were confirmed by DNA sequencing.

ELISA. GST-fusion proteins of PDZ domain-containing proteins were bound to 96-well Micro Test plates (Falcon) at 10 μg/ml in 50 mM Tris-HCl (pH 7.9). Plates were incubated overnight at 4°C, washed three times in phosphate-buffered saline (PBS) and blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at 37° C. After washing four times in PBS, serial 1:3 dilutions (starting at 200 μg/ml) of thioredoxin-SAPK3(1-367) or thioredoxin-SAPK3 (1-363) in 1% BSA/PBS + 0.1% Tween 20 (w/v)

were added and allowed to bind for 1 h at 37°C. Plates were washed four times in PBS + 0.1 % Tween 20, incubated with anti-thioredoxin antibody (1:3000, Invitrogen) for 1 h at 37° C, washed four times in PBS + 0.1 % Tween 20 and incubated with goat anti-mouse IgG-conjugated peroxidase (1:2000, Bio-Rad) for 1 h at 37° C. Plates were washed three times in PBS, followed by the addition of 100 µl of 50 mM citrate-phosphate buffer (pH 5.0) + 0.5 mg/ml o-phenylenediamine (Sigma). After 5 min the colour reaction was stopped by addition of 20 µl of 8N H_2SO_4 and absorbance at 450 or 490 nm determined using a microplate reader (Molecular Devices).

Identification of phosphorylation sites. GST-α1-syntrophin (0.5 µM) was incubated at 30° C for 1 h with activated GST-SAPK3 (2 U/ml) [23], 10 mM magnesium acetate and 100 µM [γ^{32}P]ATp in a total volume of 200 µl of 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1 mM sodium orthovanadate and 0.1% (v/v) 2-mercaptoethanol. After SDS-PAGE and autoradiography, the band corresponding to [^{32}P]-labelled α1-syntrophin was excised, digested with trypsin and the phosphopeptides generated chromatographed on a Vydac 218TP54 C_{18} column equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) and the column developed with a linear acetonitrile gradient. The flow-rate was 0.8 ml/min and fractions of 0.4 ml were collected. The two peaks of [^{32}P] radioactivity were analysed by solid and gas-phase sequencing (28) and also by electrospray mass spectrometry, to identify the peptide sequences and sites of phosphorylation. SAPK3 was assayed routinely with MBP as substrate (23). Phosphorylation of α1-syntrophin by wild-type GSTSAPK3, GST-SAPK3(1-363) and GST-L367VSAPK3 was carried out in the same manner. Reactions were stopped by the addition of 1 ml 10% (w/v) trichloroacetic acid (TCA) and, after centrifugation for 10 min at 13,000 x

g, the supernatants were discarded. The pellets were washed three times with 1 ml 25% (w/v) TCA and [^{32}p] incorporation measured by Cerenkov counting. Incorporation of phosphate into substrate was kept below 0.1 mole phosphate/mole substrate in all experiments, to ensure that initial rate conditions were met.

Immunofluorescence. Pectoral and semitendinous muscles were dissected from five adult Sprague-Dawley rats and kept at -70^0 C until use. Cryosections (10 μm) were dipped in acetone, air-dried and fixed in 2% paraformaldehyde (w/v). Following a 5 min wash in phosphate-buffered saline (PBS), sections were incubated overnight at 4° C in 10^{-7} M tetramethylrhodamine α-bungarotoxin (Molecular Probes, Inc.) diluted in PBS. Tissue sections were then washed for 15 min in PBS and fixed for 5 min in ethanol. For double-staining, tissue sections were further incubated overnight with anti-SAPK3 serum R5 (diluted 1:200). R5 was raised in a rabbit against the synthetic peptide KPPRQLGARVPKETAL (corresponding to residues 352-367 of rat SAPK3) conjugated to keyhole limpet hemocyanin. After a 30 min wash in PBS, tissue sections were incubated for 2 h at room temperature with biotinylated anti-rabbit secondary antibody (diluted 1:200, Vector Laboratories) and, following a further 30 min wash in PBS, they were incubated for 1 h at room temperature with fluorescein-avidin D (diluted 1:200, Vector Laboratories). Sections which were triple-stained were washed in PBS, blocked using the Vector blocking kit and incubated overnight at 4° C with anti-α1-syntrophin serum SYN17 (diluted 1:50) (29). Incubation with biotinylated secondary antibody and washings were done as for doublestaining and sections were then incubated for 1 h at room temperature with AMCA-streptavidin (diluted 1:50, Boehringer). Sections were mounted using Vectashield mounting medium. Immunofluorescence

was observed using a Leitz DMRD fluorescence microscope using filters for rhodamine, fluorescein and AMCA. In parallel experiments, muscle sections were single-stained with tetramethylrhodamine α-bungarotoxin, antiserum R5 or antiserum SYN17. As a control for the specificity of staining, diluted antiserum R5 was incubated with 10 μM recombinant GST-SAPK3, prior to staining. Moreover, in double- or triple-stainings, tetramethylrhodamine α-bungarotoxin and antibodies R5 or SYN 17 were alternatively omitted.

Transfection and immunoprecipitation. Full-length rat SAPK3 and human α1-syntrophin cDNAs were subcloned into the eukaryotic expression vector pSG5 and COS cells transiently transfected with 10 μg/ml plasmid DNA using DEAE-dextran chloroquine. After 48 h cells transfected with SAPK3 alone and double-transfected with SAPK3 and α1-syntrophin were lysed in 300 μl buffer (20 mM Tris-acetate, pH 7.5, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1%Triton X-100, 10mM β-glycerophosphate, 0.1% 2-mercaptoethanol (w/v), 1 mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride and 5 μg/ml leupeptin). Aliquots (100 μl) of cell lysates were incubated for 90 min at 4°C on a shaking platform with 20 μl protein A-Sepharose conjugated to μl anti-α1-syntrophin serum TROPHA. TROPHA was raised in a rabbit against the synthetic peptide ASGRRAPRTGLLELRAG (corresponding with residues 2-17 of human α1-syntrophin) coupled to keyhole limpet hemocyanin. The suspensions were centrifuged for 1 min at 13,000 rpm, the immunoprecipitates washed twice with 1 ml lysis buffer containing 0.5 M NaCl and once with 1 ml lysis buffer, followed by resuspension in gel loading buffer. Immunoprecipitates were detected with anti-α1-syntrophin serum TROPHA and anti-SAPK3 serum R5.

Results.

To identify SAPK3 substrates, we performed a yeast two-hybrid screen of a human brain cDNA library using rat SAPK3 as bait. This screen yielded two independent clones encoding residues 85-505 of α1-syntrophin. α1-Syntrophin is a peripheral membrane protein that comprises two pleckstrin homology (PH) domains, a PDZ domain and a unique carboxy-terminal domain, with the PDZ domain being inserted into the first PH domain (30-33). The related proteins β1-syntrophin and β2-syntrophin share a similar domain organisation (31-34). Syntrophins are believed to function as modular adapters which recruit signalling proteins to the dystrophin-glycoprotein complex at the plasma membrane (35). The yeast two-hybrid system was used to examine the domains that are responsible for the α1-syntrophin/SAPK3 interaction (Figure 1). Full-length α1-syntrophin interacted with SAPK3. The shortest construct that was positive when paired with SAPK3 contained the PDZ domain (residues 78-179) of α1-syntrophin. By contrast, a construct extending from the end of the PDZ domain to the carboxy-terminus of α1-syntrophin (residues 174-505) failed to interact with SAPK3, establishing that the PDZ domain of α1-syntrophin mediates the binding to SAPK3. PDZ domains are known to interact with the C-termini of proteins that have the consensus sequence -ES/TXV (36,37). The carboxy-terminus of rat SAPK3 (amino acid sequence -ETAL) (20) is similar to this consensus sequence. Deletion of the last four amino acids of SAPK3 prevented its association with α1-syntrophin, demonstrating that this sequence is necessary for the interaction (Figure 1). The syntrophin constructs were also expressed as GST-fusion proteins and their binding to thioredoxin-SAPK3 assessed by ELISA (Figure 1). As in the yeast two-hybrid system, SAPK3 bound through its carboxy-terminal four amino acids to

the PDZ domain of α1-syntrophin. Similarly, SAPK3 interacted with the PDZ domain of β1-syntrophin (Figure 1), whereas it failed to bind to the PDZ domain of neuronal nitric oxide synthase (nNOS) (Figure 1) which forms homotypic interactions with the PDZ domain of α1-syntrophin and PDZ domains 1 and 2 of postsynaptic density protein 95 (PSD95/SAP90) (38). The PDZ domain of nNOS bound to α1-syntrophin both in the yeast two-hybrid system and by ELISA (not shown).

Human α1-syntrophin contains nine S/T-P sites located outside the PDZ domain which are potential sites of phosphorylation by SAP kinases (30,31). Activated GST-SAPK3 phosphorylated GST-α1-syntrophin to 2 mol phosphate/mol protein *in vitro* and two [^{32}P]-labelled tryptic peptides were identified which corresponded to residues 198-207 and 178-197, respectively (Figure 2A). Solid and gas-phase sequencing, as well as electrospray mass spectrometry were used to identify the phosphorylated residues as serines 193 and 201, which are located between the PDZ domain and the second half of the first PH domain (Figure 1A). Initial rates of phosphorylation showed that relative to myelin basic protein α1-syntrophin is a good substrate for SAPK3, but not for other SAP kinases or for p42 MAP kinase (Table 1). SAPK3 phosphorylated α1-syntrophin at approximately the same rate as it phosphorylated myelin basic protein (MBP), its standard substrate (Table 1). Phosphorylation of α1-syntrophin by SAPK3 was dependent on the carboxy-terminal four amino acids of SAPK3, as demonstrated by three separate lines of evidence (Figure 2B-D).

α1-Syntrophin was a poor substrate for GST-SAPK3(1-363), which lacks the carboxy-terminal four amino acids, whereas MBP was an equally good substrate for both GST-SAPK3(1-363) and GST-SAPK3(1-367) (Figure

2B). Furthermore, preincubation of wild-type rat GST-SAPK3 with an antibody raised against its carboxy-terminal 16 amino acids prevented phosphorylation of α1-syntrophin, but not MBP (Figure 2C). Finally, preincubation of α1-syntrophin with synthetic peptides corresponding to the carboxy-terminal 6 or 8 amino acids of rat SAPK3 prevented phosphorylation of α1-syntrophin by GST-SAPK3 (Figure 2D).

The carboxy-terminal sequence -KETAL of mouse, rat, rabbit and zebrafish SAPK3 (20, unpublished) or -KETPL of human SAPK3 (10,21,22) is the most conserved sequence in the carboxy-terminal region of SAPK3 and differs from the prototypical consensus PDZ domain-binding sequence (36,37) by replacement of the terminal valine with leucine. We therefore investigated the ability of rat GST-L367VSAPK3 to bind and phosphorylate GST-α1-syntrophin. By ELISA, the binding of wild-type GST-SAPK3 to α1-syntrophin was similar to that of mutant GST-L367VSAPK3 (Figure 3A). The rate of phosphorylation of α1-syntrophin by GST-L367VSAPK3 was slightly faster than by wild-type GST-SAPK3 (Figure 3B). However, both mutant and wild-type SAPK3 phosphorylated α1-syntrophin to the same extent (Figure 3B). The phosphorylation of MBP by SAPK3 was unaffected by the L367V mutation (Figure 3C).

If the association of SAPK3 with α1-syntrophin is physiologically relevant, the two proteins should be co-localized *in vivo*. Both SAPK3 and α1-syntrophin are expressed at highest levels in skeletal muscle (20-22,30,31), where α1-syntrophin is associated with the sarcolemma and concentrated at the neuromuscular junction (39). We used immunofluorescence to examine the localization of SAPK3 in rat skeletal muscle. SAPK3 was found throughout the sarcolemma and was

concentrated at the neuromuscular junction, as indicated by its co-localization with α-bungarotoxin which visualizes nicotinic acetylcholine receptors at the neuromuscular junction (Figure 4). Moreover, double-staining for SAPK3 and α1-syntrophin showed extensive co-localization, both at the neuromuscular junction and throughout the sarcolemma (Figure 4). The staining was specific, as it was abolished by incubation of diluted SAPK3 antiserum with 10μM recombinant SAPK3 (not shown). For an independent assessment of the α1-syntrophin/SAPK3 interaction, the ability of SAPK3 to co-immunoprecipitate with α1-syntrophin was examined in extracts from mammalian cells co-transfected with both proteins. α1-Syntrophin and SAPK3 were co-expressed transiently in COS cells. Immunoprecipitation was carried out using an anti-α-syntrophin antibody and proteins present in the pellet immunoblotted using anti-α1-syntrophin and anti-SAPK3 antibodies. The strong signal seen for SAPK3 upon immunoprecipitation with the anti-α-syntrophin antibody indicates that α1-syntrophin existed in a complex with SAPK3 in COS cell lysates (Figure 5).

Discussion

We have now shown that SAPK3 is a protein kinase whose phosphorylation of α1-syntrophin depends on the interaction between its carboxy-terminal sequence and the PDZ domain of this substrate. The carboxy-terminal sequence of SAPK3 thus provides a mechanism both for its selective targeting to subcellular sites and for determining its substrate specificity. During vulval induction in *C. elegans*, the PDZ domain-containing protein LIN-7 is essential for localising the EGF receptor-like tyrosine kinase LET-23 to cell junctions by binding through its PDZ domain to the carboxy-terminal sequence -KETCL of LET-23 (40-42).

Similarly, protein kinase C (PKC) α is a protein kinase that is targeted to subcellular sites through the interaction of its carboxy-terminal sequence-QSAV with the PDZ domain of the PKC α-binding protein (PICK1) (43). Moreover, p70 S6 kinase has been shown to bind through its carboxy-terminal sequence to the PDZ domain of neurabin, suggesting a mechanism for localising p70 S6 kinase to nerve terminals (44).

The α1-subunits SkM1 and SkM2 of voltage-gated sodium channels from skeletal muscle and heart (45,46) have recently been shown to bind to the PDZ domain of α1-syntrophin through their carboxy-terminal sequences -KESLV [SkM1] or -RESIV [SkM2] (46,48). In skeletal muscle the interaction between SkM1 and α1-syntrophin has been proposed as a mechanism for anchoring voltage-gated sodium channels in the depths of the junctional folds of the past-synaptic membrane (46,47). At the neuromuscular junction SAPK3 is therefore likely to be anchored in close proximity to voltage-gated sodium channels.

The carboxy-terminal sequences of voltage-gated sodium channels closely resemble the carboxy-terminus -KETAL or -KETPL of SAPK3 from different species, expect that the terminal leucine is replaced by valine. However, binding of L367VSAPK3 to the PDZ domain of α1-syntrophin was found to be similar to that of wild-type SAPK3. Phosphorylation of α1-syntrophin by L367VSAPK3 was also similar to that of wild-type SAPK3. This indicates that proteins with a leucine residue at position 0 of the consensus sequence of PDZ domain-binding proteins will bind to α1-syntrophin. Mammalian type-II activin receptors are transmembrane serine/threonine protein kinases of the TGFβ receptor superfamily with the carboxy-terminal sequences -KESSL or -KESSI (49,50), suggesting that they may also be PDZ domain-binding proteins and bind to α1-syntrophin.

Although SAPK3 is expressed at highest levels of skeletal muscle, it is expressed at lower levels in many other tissues (20). It is likely that SAPK3 will be found to interact with the PDZ domains of proteins other than α1-syntrophin. Possible candidates include the PDZ domains of proteins whose binding partners have a leucine residue at position 0, such as the recently identified Veli proteins, the vertebrate homologues of LIN-7 (51). SAPK3 is unique among members of the MAP kinase family in having a carboxy-terminal PDZ domain-binding sequence. It therefore probably serves distinct physiological functions and is not a mere isoform of SAPK2a/p38. Inactivation of endogenous SAPK3 by gene targeting and/or the use of specific inhibitors will help to identify its specific functions.

Many proteins with PDZ domains localize to specialized call junctions, such as synapses and tight junctions, where they bind to the carboxy-termini of transmembrane protein, thereby creating a mechanism for positioning and clustering these proteins and for connecting them to the cytoplasmic network (52). The finding that SAPK3 co-localizes with α1-syntrophin in skeletal muscle, that it binds to the PDZ domain of α1-syntrophin and that phosphorylation of α1-syntrophin depends on this interaction identifies a novel mechanism for targeting a protein kinase to its substrates. Protein phosphorylation may be important for modulating the interactions between PDZ domain-containing proteins and their binding partners. It is also likely that additional protein kinases which interact with PDZ domains through a carboxy-terminal targeting sequence remain to be discovered.

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Table 1. Comparison of substrate specificities of different MAP kinase family members (assayed as in Figure 2).

Kinase (0.2 U/ml)	Rates of phosphorylation relative to myelin basic protein	
	α1-Syntrophin (1 μM)	MBP (1 μM)
SAPK3	100 ± 15	100
SAPK4	32 ± 9	100
SAPK2b	9 ± 4	100
SAPK2a	7 ± 2	100
MAPK	12 ± 2	100

Example 2: Alternative assays

A Scintillation Proximity Assay (SPA) system (Amersham International) is used to assess the incorporation of ^{32}P or ^{33}P radioactivity into α1-syntrophin. In this system, the sample (containing GST-SAPK3 activated by SKK1, the compound to be tested and [γ-^{32}P]ATP or [γ-^{33}P]ATP is mixed with beads comprising scintillant and antibodies that bind α1-syntrophin. Conveniently this is done in a 96-well format. The plate is then counted using a suitable scintillation counter, using known parameters for ^{32}P or ^{33}P SPA assays. Only ^{32}P or ^{33}P that is in proximity to the scintillant, i.e. only that bound to α1-syntrophin that is then bound by the antibody, is detected.

Example 3: Assay for compounds which modulate SAPK3 activity

An assay is set up with α1-syntrophin, as described in Example 1 or Example 2. Compounds are tested in the assay and those that give rise to inhibition or activation of SAPK3 are selected for further study. Compounds are optionally further tested for effects on SAPK1, SAPK2a, SAPK2b and/or SAPK4 and those that do not affect the phosphorylation of myelin basic protein by SAPK1, SAPK2a, SAPK2b and/or SAPK4 may be selected.

The compounds tested may be compounds selected on the basis of known properties, for example ability to inhibit other protein kinases, or may be part of a library of compounds assembled for testing in a variety of screens, for example in a "lead generation" screening programme. The

compounds may be natural or synthetic, and may be generated by combinatorial chemistry, as known to those skilled in the art.

The selected compounds may be used in the design of further compounds for manufacture and test, in order to develop a structure-activity relationship (SAR).

CLAIMS

1. A method of identifying a compound that is capable of modulating the interaction between (1) a protein kinase that is capable of (a) binding to a polypeptide comprising a PDZ domain and (b) phosphorylating the said polypeptide, and (2) the said polypeptide, wherein the method comprises the step of measuring the interaction between the said protein kinase and the said polypeptide.

2. A method of identifying a compound that is capable of modulating the phosphorylation of a polypeptide comprising a PDZ domain by a protein kinase wherein the protein kinase is capable of binding to and phosphorylating the said polypeptide, the method comprising the step of measuring the phosphorylation of the said polypeptide by the said protein kinase.

3. The method of claim 1 or claim 2 wherein the said protein kinase is capable of binding to the said protein comprising a PDZ domain *via* the said PDZ domain.

4. A method of identifying a compound that is capable of modulating the interaction between SAPK3 and a polypeptide comprising a PDZ domain wherein the method comprises the step of measuring the interaction between the said SAPK3 and the said polypeptide.

5. A method of identifying a compound that is capable of modulating the phosphorylation by SAPK3 of a polypeptide comprising a PDZ domain wherein the method comprises the step of measuring the phosphorylation of the said polypeptide by the said SAPK3.

6. A method of modulating the interaction between (1) a protein kinase that is capable of (a) binding to a protein comprising a PDZ domain and (b) of phosphorylating the said protein, and (2) the said protein, wherein a compound identified or identifiable by the method of claim 1 is used.

7. A method of modulating the phosphorylation of a polypeptide comprising a PDZ domain by a protein kinase wherein the protein kinase is capable of binding to and phosphorylating the said polypeptide, wherein a compound identified or identifiable by the method of claim 2 is used.

8. A method of measuring the protein kinase activity of SAPK3 wherein a polypeptide comprising a PDZ domain that is capable of being phosphorylated by SAPK3 is used as a substrate.

9. A method according to claim 8 wherein the said polypeptide comprising a PDZ domain is α1-syntrophin.

10. A method of identifying a compound capable of modulating the activity of SAPK3 wherein the phosphorylation of a polypeptide that is capable of being phosphorylated by SAPK3 and that comprises a PDZ domain is measured.

11. A method of phosphorylating a syntrophin the method comprising contacting syntrophin with SAPK3 and a suitable phosphate donor.

12. The method of claim 11 wherein the said syntrophin is α1-syntrophin.

13. A phosphorylated syntrophin wherein the syntrophin is phosphorylated on the residues equivalent to serine 193 and/or serine 201 of full-length human α1-syntrophin.

14. A phosphorylated syntrophin obtainable by the method of claim 11 or 12.

15. A polypeptide comprising the amino acid sequence of mammalian SAPK3 or a fragment, variant, derivative or fusion thereof wherein the residue equivalent to glutamate 364 of full-length rat SAPK3 is replaced or missing and/or the residue equivalent to threonine 365 of full-length rat SAPK3 is replaced or missing, and/or the residue equivalent to alanine 366 of full-length rat SAPK3 is replaced or missing and/or the residue equivalent to leucine 367 of full-length rat SAPK3 is replaced or missing.

16. A polypeptide comprising the amino acid sequence of mammalian syntrophin or a fragment, variant, derivative or fusion thereof wherein the residues equivalent to residues 1 to 77 of full-length human α1-syntrophin are deleted and/or the residues equivalent to residues 180 to 505 of full-length α1-syntrophin are deleted or the residues equivalent to residues 1 to 173 of full-length human α1-syntrophin are deleted or the residues equivalent to residues 1 to 102 of human full-length β1-syntrophin and/or the residues equivalent to residues 205 to the C-terminus of human full-length β1-syntrophin are deleted.

17. A peptide comprising the amino acid sequence KETAL or KETPL wherein the said polypeptide is not full-length mammalian SAPK3 or a fusion thereof.

18. A polynucleotide encoding a polypeptide as defined in any one of claims 15 to 17.

19. A recombinant polynucleotide suitable for expressing a polypeptide as defined in any one of claims 15 to 17.

20. An antibody reactive towards a peptide consisting of the amino acid sequence KPPRQLGARVPKETAL, PKETAL, RVPKETAL, PKETPL, RVPKETPL or ASGRRAPRTGLLELRAG wherein the said antibody is substantially non-reactive with other portions of SAPK3 or α1-syntrophin.

21. A kit of parts useful in carrying out a method according to any one of claims 1 to 12.

22. A compound identifiable or identified by the method according to any one of claims 1 to 5, 8 to 10.

23. A compound according to claim 22 or polypeptide according to any one of claims 15 to 17 or antibody that is capable of binding to a polypeptide comprising an amino acid sequence corresponding to the consensus sequence (R/K/Q)-(E/D)- (T/S)-X-(V/I/L) wherein X represents any amino acid for use in medicine.

24. A method of disrupting the localisation of SAPK3 at the neuromuscular junction or at the sarcolemma of skeletal muscle wherein a compound or a polypeptide or antibody as defined in claim 23 is used.

25. The use of compound or polypeptide or antibody as defined in claim 23 in the manufacture of a medicament for use in treating a patient in need thereof, for example a patient with a muscular disease, for example muscular dystrophy, or in need of modulation of phosphorylation of a protein comprising a PDZ domain or in need of modulation of signalling *via* an activin receptor or a voltage gated channel or in need of disrupting the localisation of SAPK3 at the neuromuscular junction or at the sarcolemma of skeletal muscle.

26. A method of identifying a phosphatase that is capable of dephosphorylating a phosphorylated syntrophin wherein a phosphorylated syntrophin according to claim 14 is used, the method comprising contacting the said phosphorylated syntrophin with a preparation that may comprise a said phosphatase and determining whether and to what extent the said phosphorylated syntrophin is dephosphorylated.

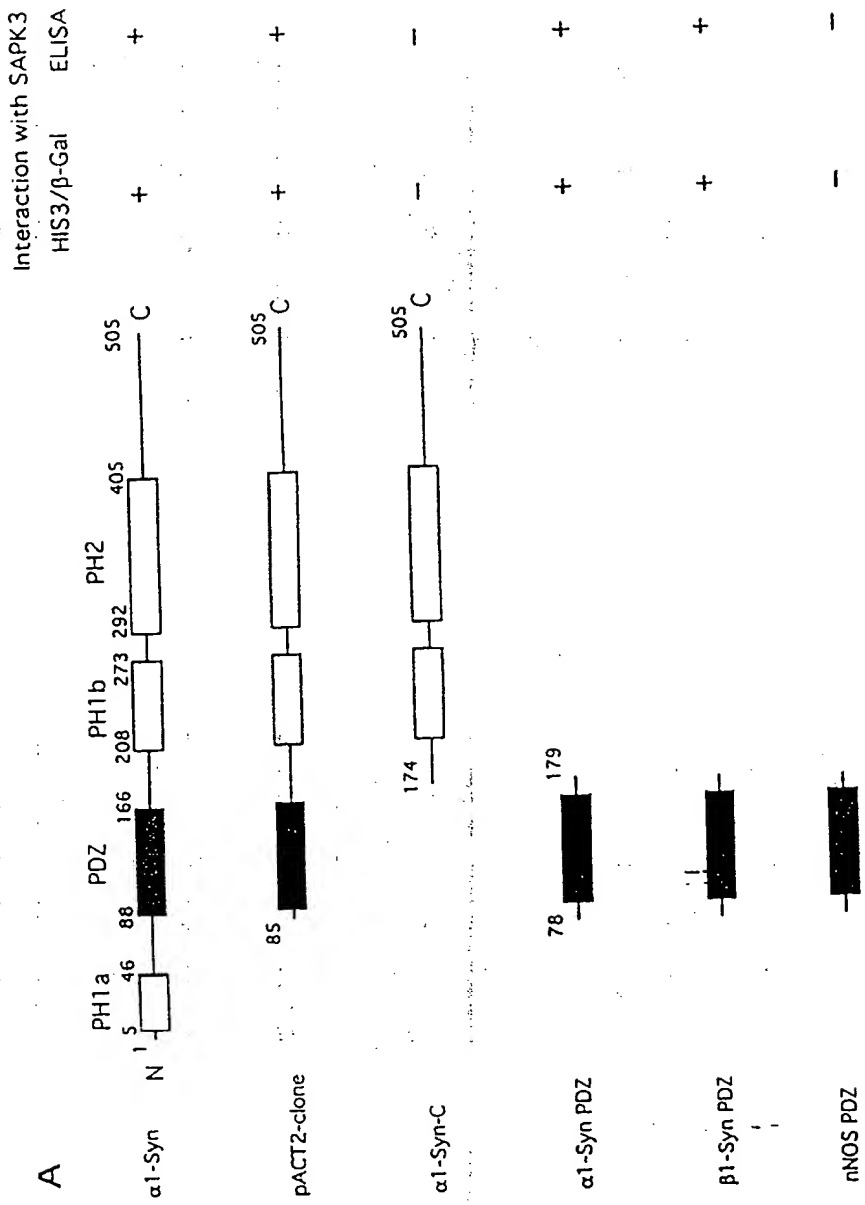
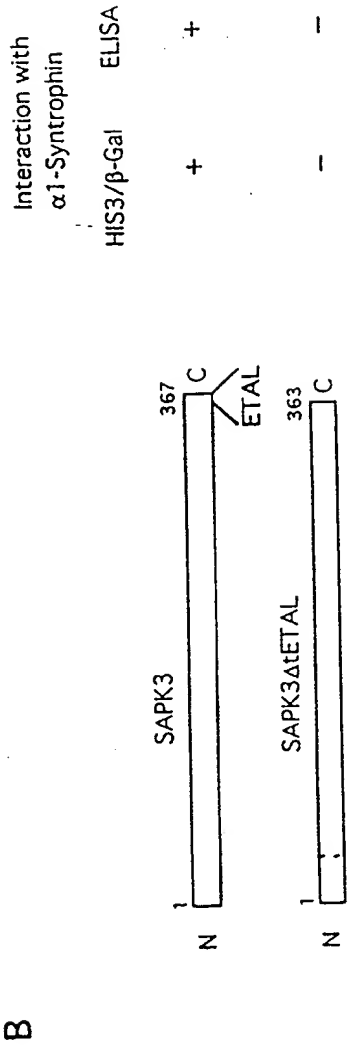


Fig 1 (page 1 of 2)



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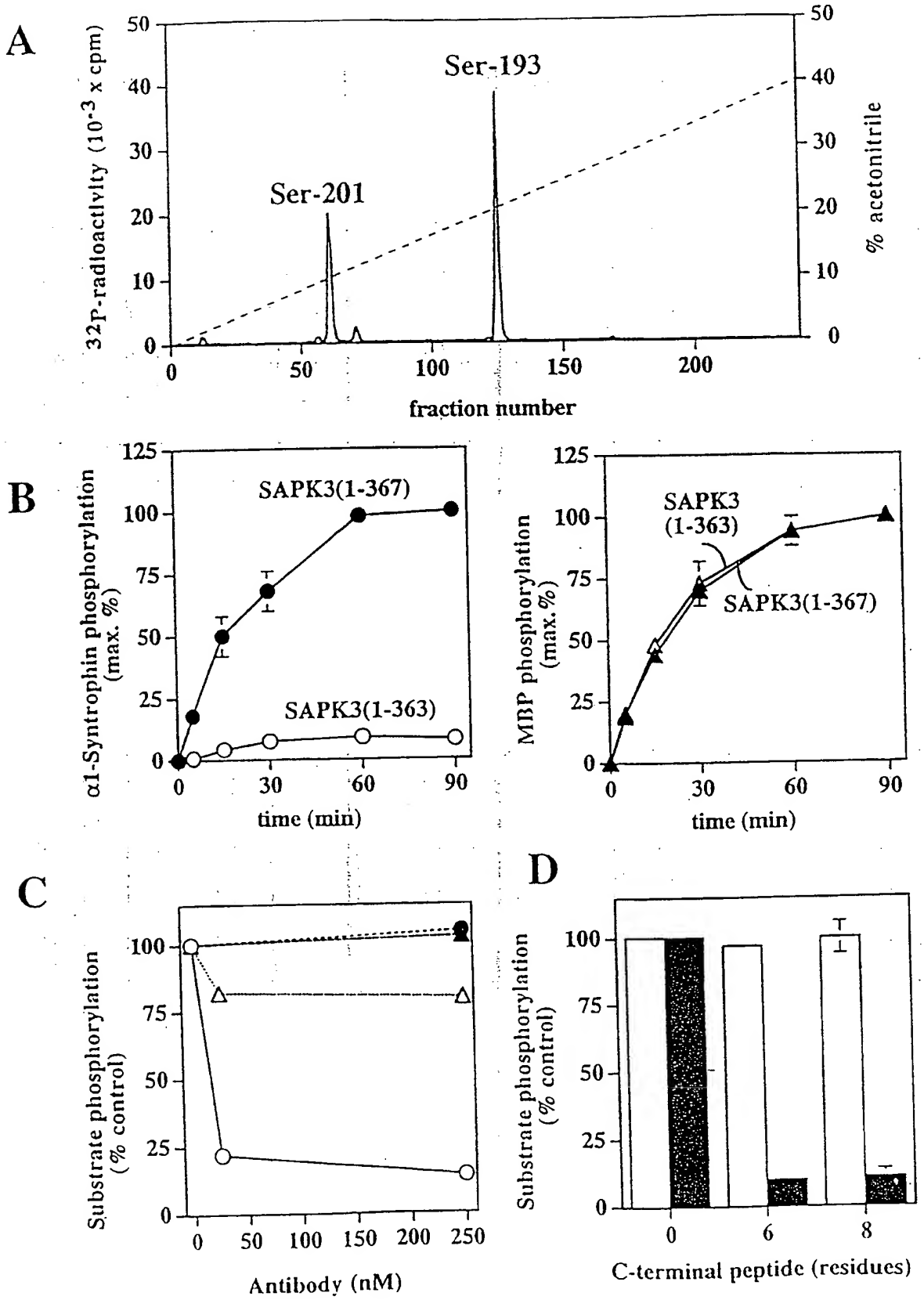
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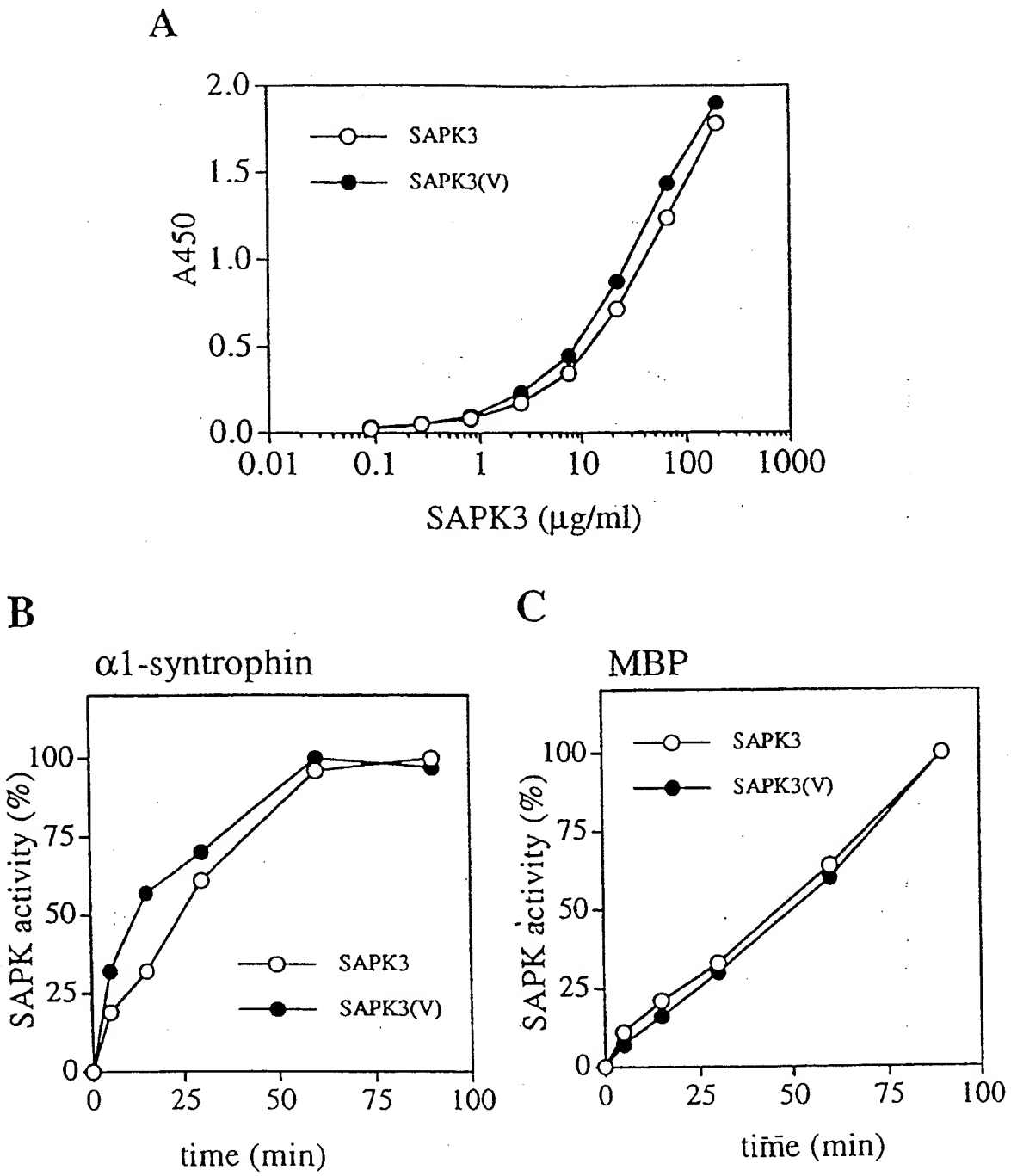
time (min)
SAPK3 (1-363)
SAPK3(1-367)
MBP phosphorylation (max.%)
time (min)
C
Substrate phosphorylation (% control)
Antibody (nM)
D
Substrate phosphorylation (% control)
C-terminal peptide (residues)

Fig 2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1.0
0.5
0.0
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0.01
0.1
1
10
100
1000
SAPK3 (µg/ml)
B
α1-syntrophin
SAPK activity (%)
100
75
50
25
0
SAPK3
SAPK3(V)
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time (min)
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time (min)

Fig 3

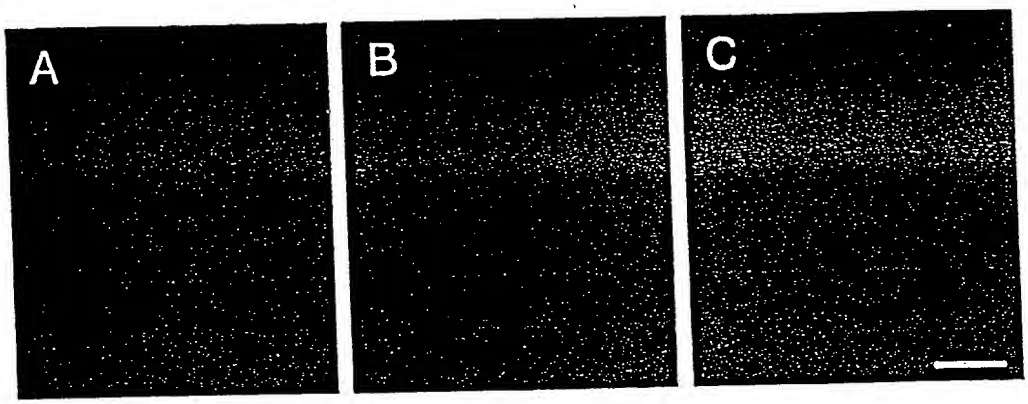
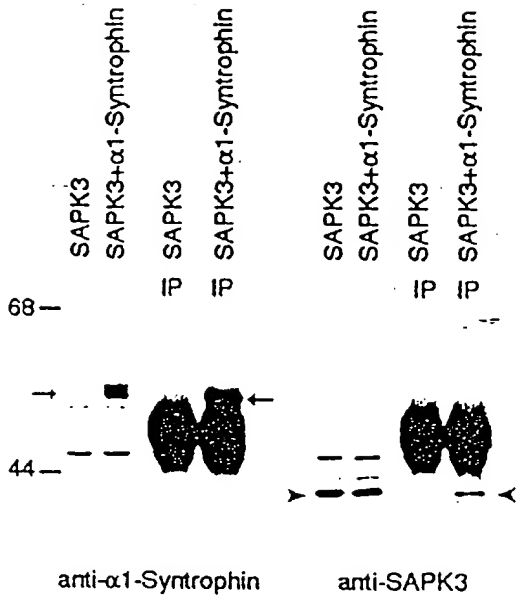


Fig 4


SAPK3
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44
anti-α1-Syntrophin
anti-SAPK3

Fig 5

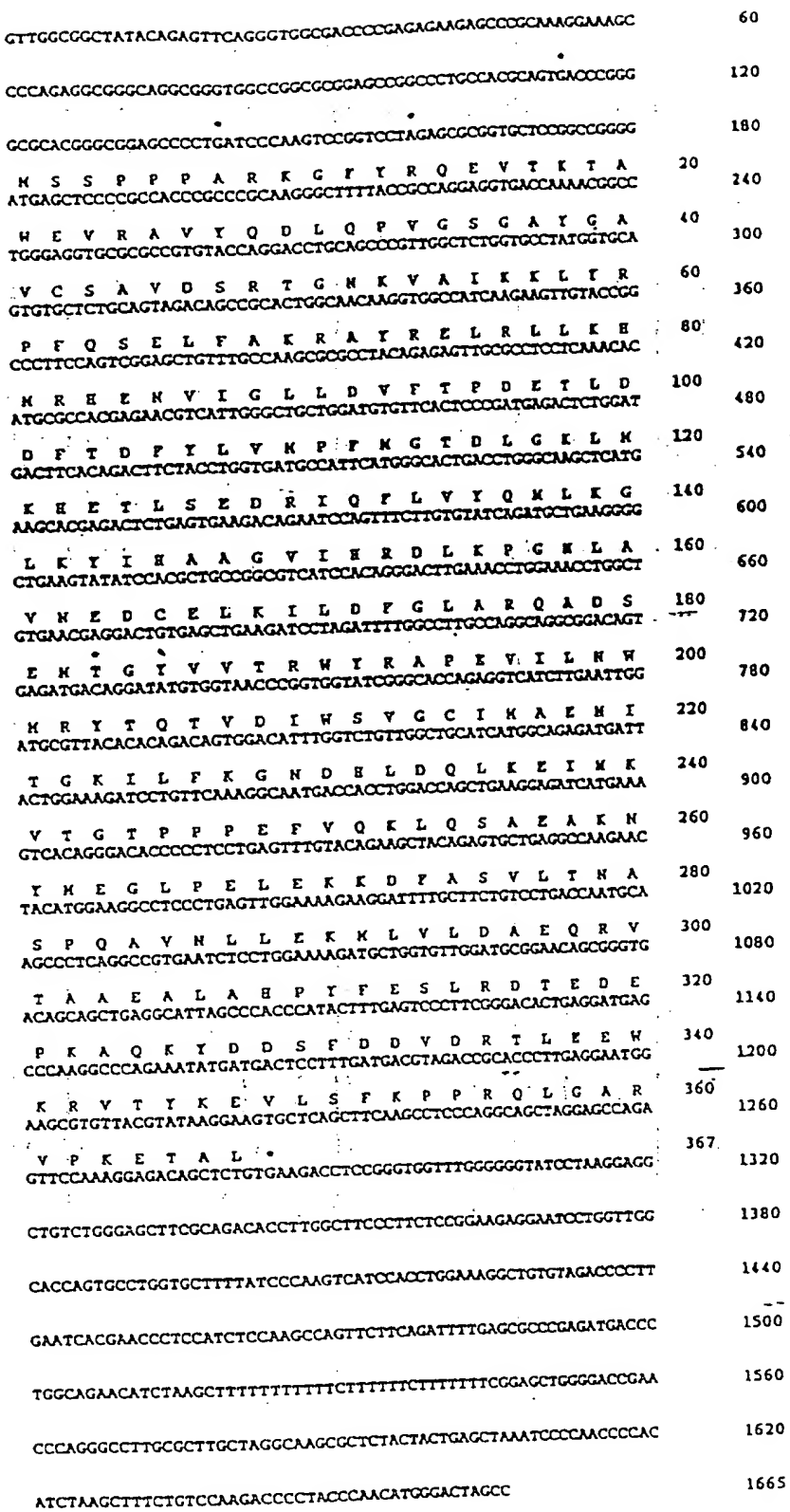
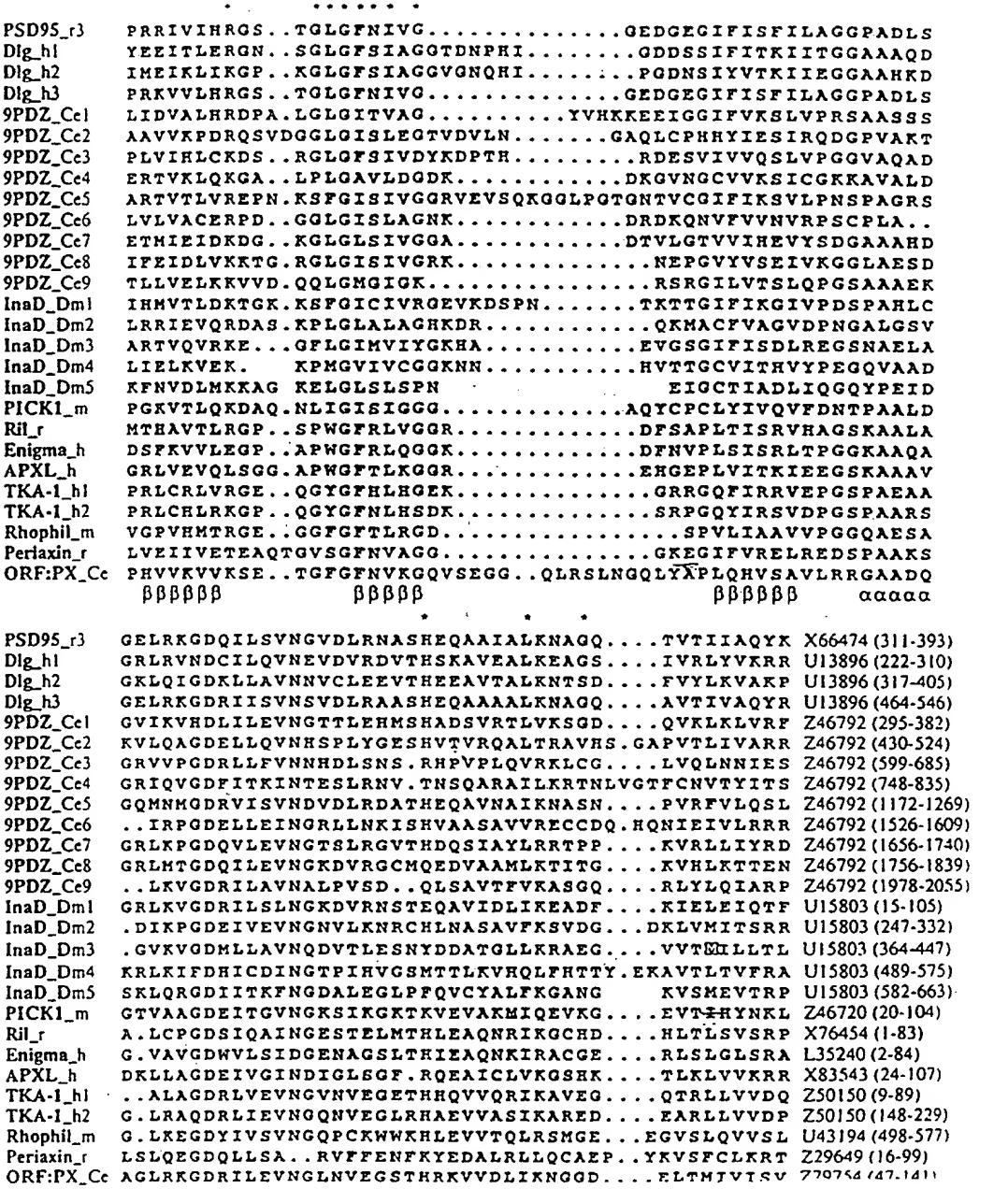


Fig 6



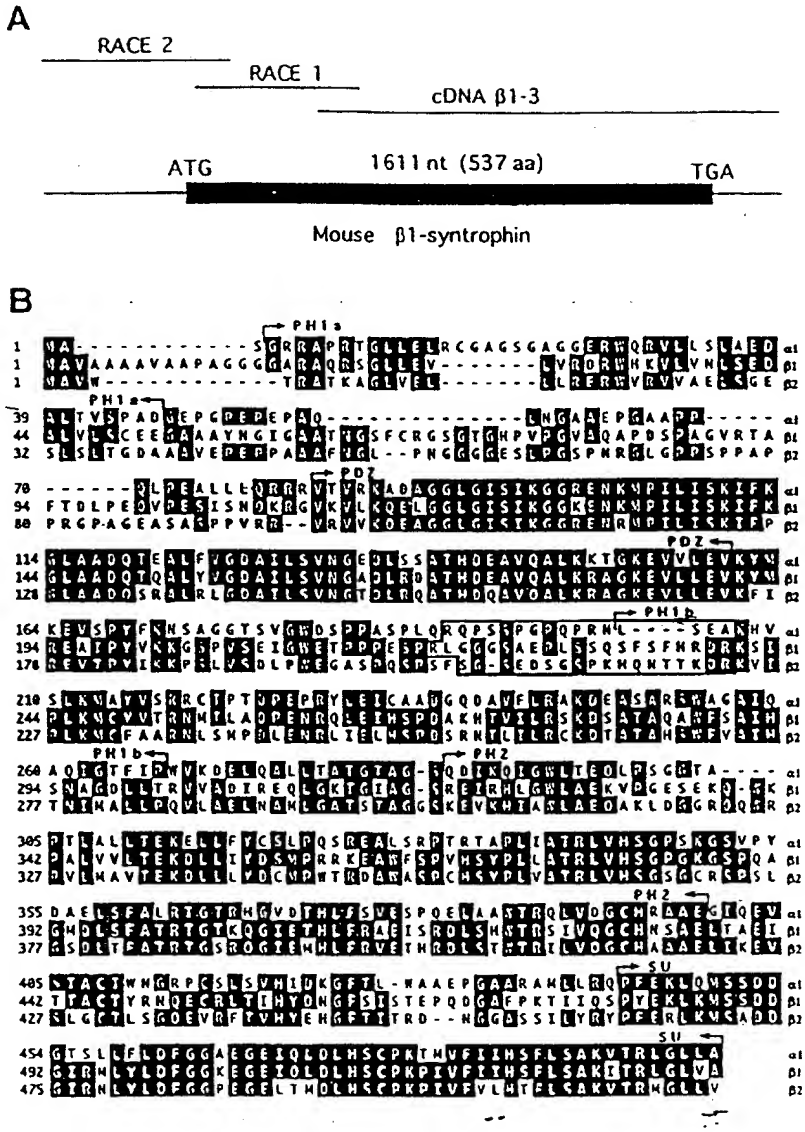
Multiple alignment of representative PDZ domain sequences, including those of two PDZ domains, rat PSD-95 PDZ3 and human Dlg PDZ3[35,36], whose tertiary structures are known, and a 9-PDZ domain containing protein ('9PDZ') from *C. elegans*.

EMBL accession codes and residue numbers follow the alignment. Insertions/deletions are denoted by dots, and the known secondary structure of PDZ domains[35,36] is shown beneath the alignment (β=β-strand, α=α-helix). Asterisks (*) above the alignment denote residues that contact the ligand in the rat PSD-95 PDZ3 structure[35]. The methionine residue substituted in *InaD*^{P215} flies that show altered light responses[32] is shown in outline. Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; h, *Homo sapiens*; m, *Mus*

Fig 7

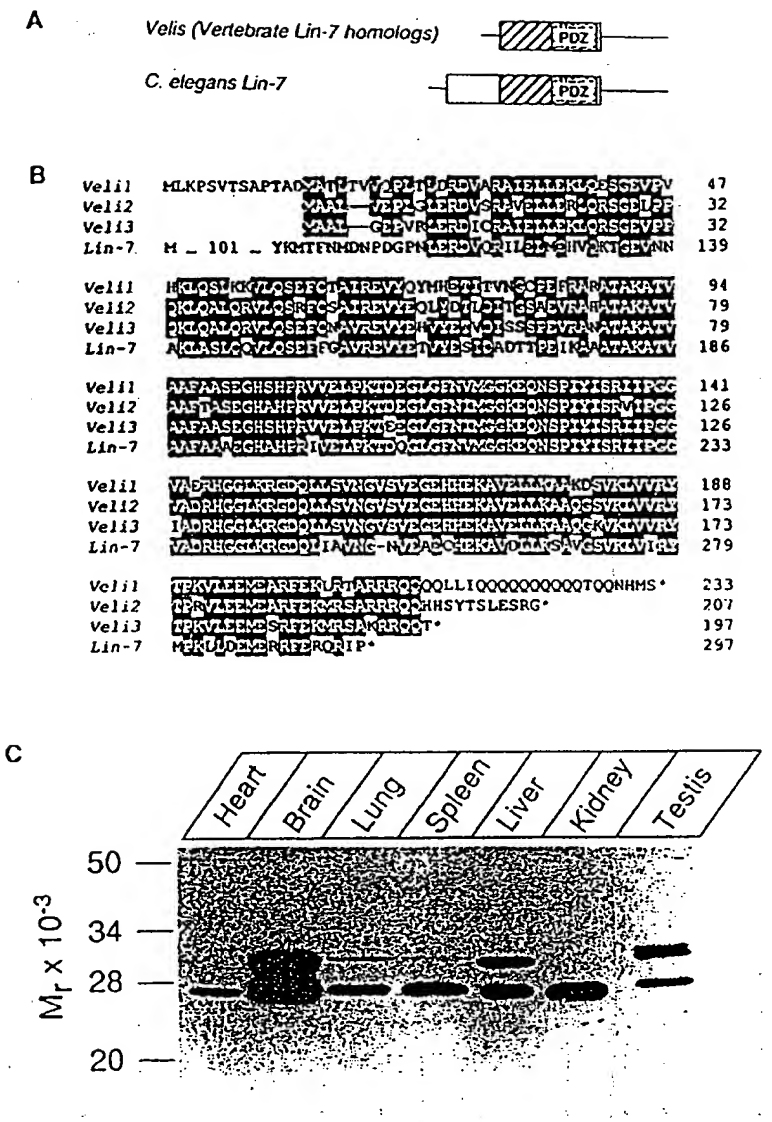
Sequence comparison of PDZ domains. The PDZ domain family is divided into classes I and II. Secondary structural elements (as defined in Fig. 3) and the variable loop are indicated. Residues that comprise the two hydrophobic binding pockets for the ligand are shown in magenta and cyan. Numbering is according to residues 489–572 of hCASK. The Gly-Leu-Gly-Phe and the *h*-Gly-*h* motifs, which comprise the carboxylate binding loop, are both shown in blue.

Fig 8



Cloning, sequence, and domain structure of *murine* β1-syntrophin sequence. (*A*) Strategy for cloning mouse β1-syntrophin and structure of the combined cDNAs, showing the coding region bounded by start and stop codons. (*B*) The deduced amino acid sequence of mouse β1-syntrophin is aligned with mouse α1- and β2-syntrophins (1, 2). Identical amino acids are shaded. The boundaries of PH (6, 19), PDZ (2), and SU (2) domains are indicated by arrows. The boxed region denotes sequences used to generate synthetic peptides for production of isoform-specific antibodies. (*C*) Schematic diagram showing the relative organization of PH, PDZ, and SU domains in syntrophins. Mouse β1-syntrophin cDNA sequence data are available from GenBank/EMBL/DBJ under the accession number U89997.

Fig 9



Characterization of Velis (*V*ertebrate *LI*N-7 Homologs)

(A) Domain structures of Velis and *C. elegans* LIN-7.

(B) Sequence alignment of human Veli1, murine Velis 2 and 3, and *C. elegans* LIN-7. Residues that are shared among the four sequences are shown in white on a black background. The PDZ domain (residues 117 to 190 in Veli1) is depicted on a lighter background than the sequences preceding or following it.

(C) Immunoblot analysis of Velis in rat tissues. Equivalent amounts of protein from the indicated tissues were probed with an antibody raised to Veli2. Immunoprecipitations and amino acid sequencing of Velis from rat brain indicated that the various bands detected all correspond to Velis (not shown). Molecular weight standards are shown on the left.

Fig 10

```
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NID         g3318652
SOURCE      human.
  ORGANISM  Homo sapiens
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            Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 3995)
  AUTHORS   Stathakis,D.G., Hoover,K.B., You,Z. and Bryant,P.J.
  TITLE     Human postsynaptic density-95 (PSD95): location of the gene (DLG4)
            and possible function in nonneural as well as in neural tissues
  JOURNAL   Genomics 44 (1), 71-82 (1997)
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  AUTHORS   Stathakis,D.G., Hoover,K.H., You,Z. and Bryant,P.J.
  TITLE     Direct Submission
  JOURNAL   Submitted (24-DEC-1996) Developmental Biology Center, University of
            California, Irvine, 4240 Biological Sciences II, Irvine, CA
            92697-2275, USA
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  AUTHORS   Stathakis,D.G., Hoover,K.H., You,Z. and Bryant,P.J.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-JUL-1998) Developmental Biology Center, University of
            California, Irvine, 4240 Biological Sciences II, Irvine, CA
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Fig 11

```
Human alpha1-syntrophin (SNT A1) mRNA, complete cds.
ACCESSION   U40571
NID         g1145727
KEYWORDS    .
SOURCE      human.
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            Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;
            Vertebrata; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 2110)
  AUTHORS   Ahn,A.H., Freener,C.A., Gussoni,E., Yoshida,M., Ozawa,E. and
            Kunkel,L.M.
  TITLE     The three human syntrophin genes are expressed in diverse tissues,
            have distinct chromosomal locations, and each bind to dystrophin
            and its relatives
  JOURNAL   J. Biol. Chem. 271 (5), 2724-2730 (1996)
  MEDLINE   96162017
REFERENCE   2  (bases 1 to 2110)
  AUTHORS   Ahn,A.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-NOV-1995) Andrew H. Ahn, Division of Genetics, HHMI
            Childrens Hosp, 300 Longwood Avenue, Boston, MA 02115, USA
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Fig 12a

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Human beta1-syntrophin (SNT B1) gene, complete cds.
ACCESSION   L31529
NID         g1066339
KEYWORDS    dystrophin; syntrophin.
SOURCE      Homo sapiens DNA.
  ORGANISM  Homo sapiens
            Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;
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REFERENCE   1  (bases 1 to 2978)
  AUTHORS   Ahn,A.H., Yoshida,M., Anderson,M.S., Feener,C.A., Selig,S.,
            Hagiwara,Y., Ozawa,E. and Kunkel,L.M.
  TITLE     Cloning of human basic A1, a distinct 59-kDa dystrophin-associated
            protein encoded on chromosome 8q23-24
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 91 (10), 4446-4450 (1994)
  MEDLINE   94240154
REFERENCE   2  (bases 1 to 2978)
  AUTHORS   Ahn,A.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-MAY-1994) Andrew H. Ahn, Division of Genetics, HHMI
            Childrens Hosp., Boston, MA 02115, USA
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                     SPRLGGSTSDPPSSQSFSFHRDRKSIPLKMCYVTRSMALADPENRQLEIHSPDAKHTV
                     ILRSKDSATAQAWFSAIHSNVNDLLTRVIAEVREQLGKTGIAGSREIRHLGWLAEKVP
                     GESKKQWKPALVVLTEKDLLIYDSMPRRKEAWFSPVHTYPLLATRLVHSGPGKGSPQA
                     GVDLSFATRTGTRQGIETHLFRAETSRDLSHWTRSIVQGCHNSAELIAEISTACTYKN
                     QECRLTIHYENGFSITTEPQEGAFPKTIIQSPYEKLKMSSDDGIRMLYLDFGGKDGEI
                     QLDLHSCPKPIVFIIHSFLSAKITRLGLVA
```

Fig 12b

```
LOCUS       HSU40572     1700 bp    mRNA            PRI       25-APR-1996
DEFINITION  Human beta2-syntrophin (SNT B2) mRNA, complete cds.
ACCESSION   U40572
NID         g1145729
KEYWORDS    .
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;
            Vertebrata; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 1700)
  AUTHORS   Ahn,A.H., Freener,C.A., Gussoni,E., Yoshida,M., Ozawa,E. and
            Kunkel,L.M.
  TITLE     The three human syntrophin genes are expressed in diverse tissues,
            have distinct chromosomal locations, and each bind to dystrophin
            and its relatives
  JOURNAL   J. Biol. Chem. 271 (5), 2724-2730 (1996)
  MEDLINE   96162017
REFERENCE   2  (bases 1 to 1700)
  AUTHORS   Ahn,A.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-NOV-1995) Andrew H. Ahn, Division of Genetics, HHMI
            Childrens Hosp, 300 Longwood Avenue, Boston, MA 02115, USA
FEATURES             Location/Qualifiers
     source          1..1700
                     /organism="Homo sapiens"
                     /db_xref="taxon:9606"
                     /chromosome="16"
                     /map="16q23-24"
     gene            21..1643
                     /gene="SNT B2"
     CDS             21..1643
                     /gene="SNT B2"
                     /note="contains two pleckstrin homology domains and a
                     domain related to both the tumor discs-large protein and
                     the zonula occludens protein; dystrophin-binding
                     intracellular membrane cytoskeletal protein"
                     /codon_start=1
                     /product="beta2-syntrophin"
                     /db_xref="PID:g1145730"
                     /translation="MRVAAATAAAGAGPAMAVWTRATKAGLVELLLRERWVRVVAELS
                     GESLSLTGDAAAAELEPALGPAAAAFNGLPNGGGAGDSLPGSPSRGLGPPSPPAPPRG
                     PAGEAGASPPVRRVRVVKQEAGGLGISIKGGRENRMPILISKIFPGLAADQSRALRLG
                     DAILSVNGTDLRQATHDQAVQALKRAGKEVLLEVKFIREVTPYIKKPSLVSDLPWEGA
                     APQSPSFSGSEDSGSPKHQNSTKDRKIIPLKMCFAARNLSMPDLENRLIELHSPDSRN
                     TLILRCKDTATAHSWFVAIHTNIMALLPQVLAELNAMLGATSTAGGSKEVKHIAWLAE
                     QAKLDGGRQQWRPVLMAVTEKDLLLYDCMPWTRDAWASPCHSYPLVATRLVHSGSGCR
                     SPSLGSDLTFATRTGSRQGIEMHLFRVETHRDLSSWTRILVQGCHAAAELIKEVSLGC
                     MLNGQEVRLTIHYENGFTISRENGGSSSILYRYPFERLKMSADDGIRNLYLDFGGPEG
                     ELTMDLHSCPKPIVFVLHTFLSAKVTRMGLLV
```

Fig 12c

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INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/GB 00/00374

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68 C12Q1/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C12Q C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	HASEGAWA M. ET AL.: "Stress-activated protein kinase-3 interacts with the PDZ domain of alphal-syntrophin" J. BIOL. CHEM., vol. 274, 30 April 1999 (1999-04-30), pages 12626-12631, XP000907312 the whole document --- -/--	1-21, 23-26

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 June 2000	30/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Pellegrini, P

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INTERNATIONAL SEARCH REPORT

Inte. ɔnal Application No
PCT/GB 00/00374

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	MADHAVAN R. ET AL.: "Phosphorylation of dystrophin and alpha-syntrophin by Ca2+-calmodulin dependent protein kinase II" BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1434, 12 October 1999 (1999-10-12), pages 260-274, XP000913465	1-12,21
A	abstract page 266, column 2, paragraph 1 -page 267, column 1, paragraph 1	13-20, 23-26
X	HOCK B. ET AL.: "PDZ-domain-mediated interaction of the Eph-related receptor tyrosine kinase EphB3 and the ras-binding protein AF6 depends on the kinase activity of the receptor" PROC. NATL. ACAD. SCI. USA, vol. 95, 1998, pages 9779-9784, XP002138507	1-12,21
A	abstract page 9782, column 2, paragraph 2 -page 9783, column 1, paragraph 2	13-20, 23-26
X	BUCHERT M. ET AL.: "The junction-associated protein AF-6 interacts and clusters with specific Eph receptor tyrosine kinases at specialized sites of cell-cell contact in the brain" JOURNAL OF CELL BIOLOGY, vol. 144, 25 January 1999 (1999-01-25), pages 361-371, XP000907350	1-12,21
A	abstract page 363, column 2, paragraph 4 -page 365, column 2, paragraph 1 page 367, column 2, paragraph 2 -page 368, column 2, paragraph 1	13-20, 23-26
X	HUBER A. ET AL.: "Phosphorylation of the InaD gene product, a photoreceptor membrane protein required for recovery of visual excitation" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 20, 1996, pages 11710-11717, XP002138975	1-12,21
A	page 11716, column 1, paragraph 2	13-20, 23-26

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 00/00374

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	SUH K S ET AL: "An avian cDNA encoding a tyrosine-phosphorylated protein with PDZ, coiled-coil, and SAM domains" GENE,NL,ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, vol. 219, no. 1-2, pages 111-123, XP004149362 ISSN: 0378-1119	1-12,21
A	the whole document	13-20, 23-26
P,X	HALL R.A. ET AL.: "G protein-coupled receptor kinase 6A phosphorylates the Na+/H+ exchanger regulatory factor via a PDZ domain-mediated interaction" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 34, 20 August 1999 (1999-08-20), pages 24328-24334, XP000907363	1-12,21
A	abstract	13-20, 23-26
X	WO 98 15618 A (MEDICAL RES COUNCIL ;COHEN PHILIP (GB); GOEDERT MICHEL (GB)) 16 April 1998 (1998-04-16) page 63, line 6-8 claims	20,23-25
A	BURNETT P.E. ET AL.: "Neurabin is a synaptic protein linking p70 S6 kinase and the neuronal cytoskeleton" PROC. NATL. ACAD. SCI. USA, vol. 95, 1998, pages 8351-8356, XP002138506 cited in the application abstract	1-21, 23-26
A	GOEDERT M. ET AL.: "Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6); comparison of its substrate specificity with that of other SAP kinases" THE EMBO JOURNAL, vol. 16, no. 12, 1997, pages 3563-3571, XP002138509 cited in the application the whole document	1-21, 23-26
A	GOEDERT M. ET AL.: "Phosphorylation of microtubule-associated protein tau by stress-activated protein kinases" FEBS LETTERS, vol. 409, 1997, pages 57-62, XP000906954 the whole document	1-21, 23-26

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 00/00374

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	COHEN P: "THE SEARCH FOR PHYSIOLOGICAL SUBSTRATES OF MAP AND SAP KINASES IN MAMMALIAN CELLS" TRENDS IN CELL BIOLOGY,XX,ELSEVIER SCIENCE LTD, vol. 7, 1 September 1997 (1997-09-01), pages 353-361, XP002053851 ISSN: 0962-8924 cited in the application the whole document	1-21, 23-26

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

...ernational application No.
PCT/GB 00/00374

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 22, 23-25 (partially)

Claims 22-25 relate to an extremely large number of possible compounds and, as a consequence, to an extremely large number of methods and uses based on these compounds. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds, methods and uses claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to polypeptides according to claims 15 to 17 and antibodies against the C-terminal sequence of SAPK3, and to methods and uses related to these polypeptides and antibodies.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte .onal Application No
PCT/GB 00/00374

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9815618 A	16-04-1998	AU 4630297 A	05-05-1998
		EP 0932666 A	04-08-1999